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EZH2 **polymorphisms and outcome of metastatic colorectal cancer patients**

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SUMMARY

Despite therapeutic innovations, metastatic colorectal cancer (mCRC) is still characterized by poor prognosis. Few molecular markers are available to predict progression risk and to help therapeutic decisions. Polycomb group genes (PcGs) are epigenetic modifiers involved in tumor suppressor gene silencing. EZH2 is a PcG member that mediates gene silencing through histone-H3 lysine-27 methylation. In CRC, EZH2 over-expression is associated to shorter survival. Recently, 4 *EZH2* single nucleotide polymorphisms (SNPs) have been characterized: the present study aimed at evaluating the correlation between EZH2 SNPs and outcome parameters in mCRC patients.

DNA was extracted from blood samples of 110 mCRC patients treated with first-line FOLFIRI plus bevacizumab, and 104 mCRC patients treated with FOLFIRI. Genotyping was performed by Real-Time PCR. Allelic variant distribution was used to predict objective response, progression-free survival (PFS) and overall survival (OS). EZH2 mRNA levels were evaluated on lymphocytes of a parallel cohort of 50 radically resected stage II or III CRC patients.

One allelic variant (rs3757441 C/C *vs*. C/T or T/T) was significantly associated to shorter PFS and OS in both mCRC patient cohorts receiving first-line FOLFIRI (with or without bevacizumab). At multivariate analysis, the same variant resulted an independent predictor of both PFS and OS ($p<0.05$). Among the 50 patients analysed for EZH2 expression and genotyped for EZH2 rs3757441 SNP, mRNA levels were significantly higher in patients harbouring the C/C genotype with respect to C/T and T/T ($p<0.05$), with no difference between C/T and T/T genotypes.

Our results indicate that an *EZH2* SNP may be useful to predict PFS and OS in mCRC patients treated with first-line FOLFIRI (with or without bevacizumab).

KEY WORDS

Bevacizumab, Cancer stem cell, Colorectal cancer, EZH2, FOLFIRI, Polycomb, Single nucleotide polymorphisms

CHAPTER 1. *INTRODUCTION*

1.1. Treatment of metastatic colorectal cancer: state of the art

Colorectal cancer (CRC) still represents the third most common malignancy and the second leading cause of cancer death in Western countries[1]: about 30.000 new cases and over than 15.000 deaths have been recorded in Italy in 2000[2]. Even if in the 90% of the cases a radical resection of the primary tumor is possible, 25% of patients present with metastatic disease at diagnosis and 50% die from systemic disease[3].

In the last decade, great advances have been achieved in the treatment of metastatic CRC (mCRC): thanks to the availability of a greater number of effective cytotoxic and targeted agents and to the higher percentage of patients who undergo radical resection of metastases, overall survival (OS) has approached 24 months in most recent trials, and definitive cure of the disease is a realistic aim of treatment in at least 10-15% of the patients[4,5]. Moreover, together with the introduction of new drugs into clinical practice, new strategies have been developed, and therapeutic approach toward mCRC patients is growingly individualized according to the goal of treatment itself (palliation, long-term OS or cure)[6,7]. In the following paragraphs we will discuss the current data supporting the use of cytotoxic and targeted agents in the treatment of mCRC and review the role of the available prognostic and predictive biomarkers in individualising treatment algorithm in different patient subsets.

1.1.1. Role of chemotherapy in metastatic colorectal cancer

For over than 30 years 5-fluorouracil (5-FU) has been the only available treatment for which reliable data supported a role in mCRC: a meta-analysis[8] of various randomized trials has demonstrated that 5-FU improves OS and quality of life (QoL) of mCRC patients compared to best supportive care (BSC) alone and a randomized trial demonstrated that the advantage is greater when chemotherapy is started early in the course of the disease, before the onset of symptoms[9].

The introduction of irinotecan and oxaliplatin improved the antitumor activity and the efficacy of chemotherapy in this disease[7]. The combinations of irinotecan plus either infusional or bolus 5-FU/leucovorin (LV) (FOLFIRI and IFL, respectively) and oxaliplatin plus infusional 5-FU/LV (FOLFOX) have demonstrated increased activity and efficacy compared with 5-FU/LV alone in randomized studies[10,11,12,13]. Of interest, phase III studies comparing irinotecan plus 5-FU/LV with 5-FU/LV alone suggested that a more active treatment administered upfront can prolong OS, even if active second-line therapies are offered to patients progressing on 5-FU/LV. Furthermore, studies with oxaliplatin plus 5-FU/LV have indicated that a highly active first-line chemotherapy regimen may permit, in a small subgroup of initially unresectable mCRC patients, a secondary radical surgical approach on metastases after response to chemotherapy, and that approximately 30% to 40% of operated patients will survive without evidence of disease for more than 5 years[14,15]. Therefore, taken together these data indicate that in mCRC a more active first-line treatment can be more effective, and a meta-analysis of 25 randomized trials of first-

line treatment also supports the relationship between tumor response to first-line chemotherapy and OS[16].

More recently, a randomized study by the GERCOR group[17] assigned 220 untreated mCRC patients to receive first-line FOLFIRI followed by FOLFOX-6 at progression (arm A), or the reverse (arm B). Both sequences achieved similar activity and efficacy, and, of interest, median OS was 21.5 months in arm A and 20.6 months in arm B, which are among the highest survival times reported up to now in any randomized study of chemotherapy alone in mCRC. This study suggests that the exposure of mCRC patients to all the three most active agents (i.e. 5-FU/LV, irinotecan and oxaliplatin) is associated with best survival outcome. In addition, a study by Goldberg et al.[18] demonstrated the superiority of the FOLFOX-4 regimen to IFL and confirmed the importance of the three-drug exposure, considering that in the IFL arm only 24% of patients could receive oxaliplatin as second-line treatment, while in the FOLFOX-4 arm 60% of patients were able to receive salvage treatment with irinotecan. These data are in line with the results of a recent pooled analysis of seven phase III trials demonstrating that OS is correlated with the proportion of patients who received all the three active drugs in the course of their disease, but not with the proportion of patients who received any second-line therapy[19].

Another relevant advance in the management of mCRC is the availability of the oral fluoropyrimidine capecitabine. A meta-analysis of the studies comparing capecitabine plus oxaliplatin (CAPOX) with FOLFOX demonstrated that capecitabine is non-inferior to 5-FU in terms of PFS and OS, but results in a significantly lower RR[20]. Conversely, the results reported with capecitabine plus irinotecan (CAPIRI) are controversial, underlining that oral chemotherapy needs

active patient management. In fact, the EORTC 40015 trial[21] was prematurely suspended due to alarming safety concerns: CAPIRI reported an unacceptably higher rate of grade 3-4 diarrhoea compared to FOLFIRI (37% *vs.* 13%), with an increased incidence of treatment-related deaths (5 *vs.* 2 patients). As a consequence, both PFS (5.9 *vs.* 9.6 months) and OS (14.8 *vs.* 19.9 months) were shorter with CAPIRI. In the BICC-C study[22,23] CAPIRI reported shorter median PFS when compared with FOLFIRI (5.8 *vs.* 7.6 months; HR=1.36, p=0.015) and higher rates of severe events, particularly diarrhoea (47.5% *vs.* 13.9%) and dehydration (19.1% *vs.* 5.8%). On the contrary, the CAIRO trial[24] completed accrual and demonstrated an acceptable rate (26%) of grade 3-4 diarrhoea. All these trials tested the same CAPIRI schedule, so such differences in tolerability cannot be merely explained by differences in the dose intensity of chemotherapy. One possible confounding factor in the EORTC 40015 and BICC-C studies is represented by a second randomisation to either celecoxib or placebo, since coxibs have been associated with an increased risk of toxicities (particularly cardiovascular thrombotic events) in CRC patients: however, a causative role of celecoxib in explaining the observed high rate of severe diarrhoea appears unlikely since this agent seems to protect against irinotecan-induced mucosal injury[25].

In order to expose all patients to 5-FU/LV, irinotecan and oxaliplatin (thus overcoming the risk not to receive active cytotoxics in second-line in case of rapid disease progression and patient conditions deterioration) and in order to improve the activity of treatment (thus potentially increasing the rate of secondary surgery of metastases[26]), the Gruppo Oncologico Nord Ovest (G.O.N.O.) developed the triplet regimen named FOLFOXIRI which proved to be feasible with acceptable toxicities

and promising activity and efficacy in phase I-II studies[27,28]. When compared with FOLFIRI in a randomized phase III trial[29], FOLFOXIRI confirmed to be feasible and resulted in higher RR (66% *vs.* 41%, p=0.0002) and radical resection rate of metastases (15% *vs.* 6% of patients, p=0.033). Moreover, at a median follow-up of 18.4 months 216 patients have progressed and median PFS and OS were significantly longer in the FOLFOXIRI arm (9.8 *vs.* 6.9 months, p=0.0006 and 22.6 *vs.* 16.7 months, p=0.032, respectively) with a HR of 0.60 in favour of FOLFOXIRI. Finally, the rate of early progressions (within 6 months from treatment onset) was significantly lower in the FOLFOXIRI arm (18% *vs.* 45%, p<0.0001). A recent update of the trial[30] confirmed the PFS (median: 9.8 *vs.* 6.8 months, HR=0.59, p<0.0001) and OS (median: 23.4 *vs.* 16.7 months, HR=0.74, p=0.026; 5-year survival rate: 15% *vs.* 8%) benefit for the triplet at a median follow up of 60.6 months. The authors concluded that the FOLFOXIRI regimen is feasible with manageable toxicities also in a multicenter setting: the incidence of grade 3-4 neutropenia and grade 2-3 peripheral neurotoxicity is increased with FOLFOXIRI, but febrile neutropenia, diarrhoea and other toxicities are comparable to FOLFIRI. As recently reported by the same group, the benefit derived from the triplet is not entirely due to the increased rate of secondary radical surgery: in fact, FOLFOXIRI retained its superiority also when resected patients were excluded from the analysis, confirming a better palliative effect in unresectable mCRC than FOLFIRI. Moreover, such an aggressive strategy does not harm the feasibility and the efficacy of salvage therapies with the same agents used in first-line. The G.O.N.O. group conducted a phase I-II trial in order to evaluate capecitabine instead of 5-FU in a triplet regimen (XELOXIRI), but the change from infusional to oral fluoropyrimidine resulted in

increased rate of severe diarrhoea, confirming that capecitabine is not a preferable alternative to 5-FU in both irinotecan-containing doublet and triplet regimens.

Another phase III trial by the Hellenic Oncology Research Group (H.O.R.G.) evaluated the triple combination in the first-line treatment of mCRC patients[31]. Results demonstrated a non-significant trend for superiority for the FOLFOXIRI regimen compared to FOLFIRI, both in terms of activity (RR: 33.6% *vs.* 43%; secondary R0 metastasectomy: 4% *vs.* 10%) and efficacy (median TTP: 6.9 *vs.* 8.4 months; median OS: 19.5 *vs.* 21.5 months). These apparent discrepancies between the G.O.N.O. and H.O.R.G. trials can be explained by differences in treatment schedules and study populations. In fact, the G.O.N.O. regimen allows to avoid the 5-FU bolus administration and to administer a higher dose intensity of 5-FU, irinotecan and oxaliplatin, compared with the H.O.R.G. schedule (1600 mg/sqm/week *vs.* 1000 mg/sqm/week; 82.5 mg/sqm/week *vs* 75 mg/sqm/week; and 42.5 mg/sqm/week *vs.* 32.5 mg/sqm/week, respectively). As regards patient characteristics, patients aged more than 75 years were not included in the G.O.N.O. trial and if aged 70-75 an ECOG PS of 0 was required, while the H.O.R.G. study had no upper age limit (age range reached 82 years in the FOLFOXIRI arm) and elderly (more than 70-year old) patients with poor PS (1-2) were eligible: indeed, study population in the H.O.R.G. trial was older (median age: 66 *vs.* 62 years, respectively) and with a poorer performance status (ECOG PS 0/1/2: 36/53/11% in the H.O.R.G. trial *vs.* 61/37/2% in the G.O.N.O. trial). All these factors could have contributed to the different results of the triple regimens and underlined the need for adequate selection of fit patients when intensive chemotherapy is administered. Indeed, the potentials of the upfront triple strategy have been confirmed by the meta-analysis of

the G.O.N.O. and H.O.R.G. trials reported by Golfinopoulos et al.[32], who demonstrated significant advantages for FOLFOXIRI compared to FOLFIRI both in terms of disease progression and survival.

Masi et al.[33] recently published the results of a retrospective pooled analysis on 196 patients treated with first-line FOLFOXIRI in order to evaluate the long-term outcome of patients radically resected after chemotherapy. Thirty-seven (19%) could undergo a secondary R0 surgery on metastases (complete pathological response has been achieved in 4 patients) after a median of 5.5 months of treatment. The authors reported no intra-operative or post-operative mortality and a low rate of peri-operative complications (27% of cases), all of which were transient and resolved completely. After a median follow-up of 67 months, 5-year and 8-year OS were 42% and 33%, respectively. At 5 years, 29% of the resected patients were free of progression. Such results demonstrated the feasibility and the efficacy of FOLFOXIRI as conversion therapy and were consistent with the observation of Folprecht et al.[26] that more active first-line treatments result in a higher secondary resection rate of metastases.

If the abovementioned results support the role of a more intense first-line approach, several randomized phase III trials addressed the question whether all mCRC patients should receive upfront combination chemotherapy. In the CAIRO study[24], 820 patients were randomized to first-line capecitabine, second-line irinotecan, and third-line CAPOX or first-line CAPIRI and second-line CAPOX. The trial was powered to detect a superiority of the combination over single-agent based on an anticipated median OS of 14.0 *vs.* 17.5 months, but did not demonstrate any difference in OS, the primary end point (16.3 months for the sequential treatment

group *vs.* 17.4 months for the combination group; HR 0.92, 95%CI 0.79-1.08, p=0.3281), despite increased RR (41% *vs.* 20%, p<0.0001) and PFS (median: 7.8 *vs.* 5.8 months; HR 0.77, 95%CI 0.67-0.89; p=0.0002) being reported with the upfront combination. The FOCUS trial[34] randomized over 2100 patients to three different strategies: 5-FU/LV followed by irinotecan (strategy A); 5-FU/LV followed by FOLFIRI/FOLFOX (strategy B); first-line FOLFIRI/FOLFOX followed by the reverse regimen at progression (strategy C). FOCUS aimed to show non-inferiority of the single-agent approach. Again, the authors did not report OS differences among study arms (strategy C *vs.* B, median: 15.9 *vs.* 15.1 months; HR 1.06, 90%CI 0.97-1.17), while higher RR (5-FU/LV *vs.* FOLFIRI or FOLFOX: 28% *vs.* 49% or 57%, p<0.001) and PFS (5-FU/LV *vs.* FOLFIRI or FOLFOX: 6.3 *vs.* 8.5 or 8.7 months, p<0.001) were achieved with combination therapy, at the price of increased but acceptable toxicity: data from FOCUS therefore exclude a reduction of more than 5% in 2-year survival or a difference in median OS of more than 2.3 months with the upfront use of single-agent chemotherapy. Even though both CAIRO and FOCUS reported median OS in the range of 13.9-17.4 months, which is less than expected in patients treated with a sequence of doublet regimens (approximately 20 months), several factors contribute to explain this finding: the poor prognosis of the patients enrolled (in fact, potentially resectable patients were excluded in the FOCUS trial) and the small proportion of patients who received all the three active agents after initial treatment with monotherapy (in the range of 19-55%).

Among elderly or unfit patients, the recently published FOCUS-2 trial[35] demonstrated that the addition of oxaliplatin to fluoropyrimidine increases RR, with a trend toward higher PFS and equal OS, compared with single-agent

fluoropyrimidine. After comprehensive health assessment, 459 patients were randomly assigned to one of the following arms: 5-FU/LV (group A); oxaliplatin and 5-FU (group B); capecitabine (group C); or oxaliplatin and capecitabine (group D). The trial investigated whether the addition of oxaliplatin ([A *vs.* B] + [C *vs.* D]) to fluoropyrimidine is beneficial in terms of PFS and whether the substitution of 5-FU with capecitabine ([A *vs.* C]+[B *vs.* D]) improves QoL, as assessed by change from baseline to 12 weeks in global QoL. Final results demonstrate that a non-significant improvement in median PFS can be achieved with the upfront combination regimen (5.8 *vs.* 4.5 months; HR 0.84, 95%CI 0.69-1.01, p=0.07), while replacing 5-FU with capecitabine does not result in improved global QoL (56% patients receiving 5-FU reported improvement in global QoL compared with 56% receiving capecitabine): however, the incidence of grade 3 or worse toxicities, while not significantly increased with oxaliplatin (38% *vs.* 32%, p=0.17), was higher with capecitabine than with 5-FU (40% *vs.* 30%, p=0.03). Again, even in FOCUS-2 OS did not differ between monotherapy and combination groups (HR 0.99, 95%CI 0.81-1.18, p=0.91).

Taken together, these data underline a key issue in mCRC patient management, i.e. the importance to target the intensity of first-line chemotherapy on the basis of both patient and disease characteristics and the aim of treatment. In fact, if comorbidities or advanced age might impair the feasibility of more intense chemotherapy in some patients, disease burden might require (or not) the need for a rapid tumor response in others. Again, in selected cases a more active treatment might be the best choice to achieve enough tumor shrinkage and allow a radical secondary resection of metastases. We have convincing evidence that an upfront combination regimen achieves superior RR, secondary surgery of metastases rate and

PFS at the price of acceptable toxicity: for these reasons, patients with potentially resectable disease or with high tumor burden or disease-related symptoms may benefit from a more active combination regimen. On the other hand, data from phase III trials support the use of sequential single-agent chemotherapy in those cases with more indolent disease course, when the objective is prolonging OS without impairing QoL with consistent toxicities.

If the intensity of the upfront strategy may differ in different patient subgroups, however, several randomized trials convincingly demonstrated that scheduled treatment duration or pauses after a predefined period of treatment do not impair results in terms of OS and might contribute to improve treatment tolerability. The OPTIMOX-1 trial[36] was the one of the first large study suggesting that prolonging combination chemotherapy is not necessary in mCRC: investigators randomized 620 patients to FOLFOX-4 until progression or FOLFOX-7 for 12 weeks followed by 5-FU/LV (with oxaliplatin reintroduced at progression). The median duration of disease control (DDC, the primary end point, defined by the authors as PFS, or, if FOLFOX was reintroduced, addition of the initial PFS and the PFS of the reintroduction, except in case of progression at the first evaluation after FOLFOX reintroduction) did not differ between the two arms (9.0 *vs.* 10.6 months, HR 0.99, 95%CI 0.81-1.15, p=0.89) and also PFS and OS times were comparable between the continuous and the maintenance treatment arms (median PFS: 9.0 *vs.* 8.7, HR 1.06, 95%CI 0.89-1.20, p=0.47; median OS 19.3 *vs.* 21.2, HR 0.93, 95%CI 0.72-1.11, p=0.49), as was RR (58.5% *vs.* 59.2%, p=NS). Among patients allocated to maintenance 5-FU treatment, there was a trend toward reduced incidence of grade 3 oxaliplatin-related neurotoxicity (13.3% *vs.* 17.9%, p=0.12).

After this pivotal experience, two trials tested chemotherapy-free intervals during first-line therapy. The randomized phase II OPTIMOX-2 study[37] compared stop-and-go FOLFOX-7 (as tested in OPTIMOX-1) with FOLFOX-7 for 3 months followed by an observation period of 3 months (with the same regimen reintroduced at progression): the median DDC (primary end point) and median PFS were significantly longer in the maintenance arm (13.1 and 8.6 months, respectively) than in the chemotherapy-free interval arm (9.2 and 6.6 months, respectively) (HR=0.71, p=0.046 for DDC comparison; HR=0.61, p=0.0017 for PFS comparison), and a trend toward decreased OS was observed in the experimental arm (median: 19.5 *vs.* 23.8 months, HR 0.88, p=0.42; 2-year survival rate: 39.4% *vs.* 50%). Even though with several limitations due to the study design and sample dimension, OPTIMOX-2 seems to suggest that a maintenance treatment with a fluoropyrimidine may help in maximizing the benefit of upfront combination regimens, while a complete interruption of chemotherapy may be detrimental.

Similar data have been reported by Maughan et al.[38] who have recently published the final results of the COIN trial. Two of the three arms of the trial answered the question whether intermittent combination chemotherapy (XELOX or mFOLFOX for 12 weeks, than stopped and restarted at disease progression for a further 12 weeks of treatment) is non-inferior in terms of OS to continuous combination chemotherapy until progression. Investigators randomized 1630 patients and demonstrated that median OS achievable with intermittent chemotherapy is non-inferior to the one reported by continuous treatment (14.4 *vs.* 15.8 months; HR 1.084, 80%CI 1.008-1.165) (according to the study design, however, results reliably exclude a detriment larger than 10 weeks in median OS). Intermittent chemotherapy

allowed a median time interval free from chemotherapy of 3.7 months and significantly reduced toxicity, particularly grade 3 or more peripheral sensory neuropathy.

More convincingly, results of the recently published phase III GISCAD trial[39] suggest that comparable outcome can be achieved with continuous and intermittent (2 months on, 2 months off) FOLFIRI in first-line: among the 337 randomized patients, author reported equal OS results (primary endpoint) (18 months in the intermittent chemotherapy arm *vs.* 17 months in the continuous chemotherapy arm, HR 0.88, 95%CI 0.69-1.14). Also PFS was comparable in the two groups (6 months in both, HR 1.03, 95%CI 0.81-1.29), and no difference was reported even in terms of RR (34% *vs.* 42%, p=0.192). The median chemotherapy-free period in the intermittent arm was 3.5 months, in line with the data reported by COIN investigators with an oxaliplatin-based regimen. In the Italian trial the incidence of grade 3-4 toxicity was similar between arms, but this was not unexpected since main adverse events were represented by acute toxicities such as myelosuppression, fever and diarrhoea, rather than cumulative toxicity (such as oxaliplatin-induced neurotoxicity).

1.1.2. Role of bevacizumab in metastatic colorectal cancer

Vascular endothelial growth factor (VEGF) plays a crucial role in the development of new blood vessels in both healthy tissues and tumors. Its effect is mainly mediated through binding to the VEGF receptor-2, found predominantly on

the surface of the vascular endothelial cells. Induction of the intracellular tyrosine kinase activity of receptor by VEGF binding triggers the phosphorylation of a multitude of proteins with a subsequent cascade of intracellular signalling pathways[40]. VEGF plays a number of key roles in the pathogenesis of cancer: through excessive and deregulated angiogenesis, not only it allows the tumor to embark upon its exponential growth phase, but also provides an exit route for haematogenous metastases and allows them to establish themselves in distant organs[41].

Bevacizumab is a recombinant humanized monoclonal IgG1 antibody that binds to and inhibits the biologic activity of human VEGF in *in vitro* and *in vivo* assay systems. Bevacizumab contains human framework regions and the complementarity-determining regions of a murine antibody that binds to VEGF[42].

Hurwitz et al.[43] conducted a randomized phase III trial evaluating the addition of bevacizumab to first-line irinotecan-based treatment of mCRC patients. A total of 813 patients were randomly assigned to receive bolus IFL plus placebo, bolus IFL plus bevacizumab, or 5-FU/LV plus bevacizumab. Enrolment in the latter arm was discontinued, as pre-specified, when the toxicity profile of bevacizumab in combination with the bolus IFL regimen was deemed acceptable. The addition of bevacizumab to IFL significantly improved RR (44.8% *vs.* 34.8%; p=0.004), PFS (10.6 months *vs.* 6.2 months; HR 0.54, p<0.001) and OS (20.3 months *vs.* 15.6 months; HR=0.66, p<0.001), the primary end point of the trial. In terms of toxicity, adding bevacizumab to chemotherapy significantly increased the incidence of grade 3 hypertension (11% *vs.* 2.3%, p<0.01), but interestingly it did not impact significantly upon the rates of proteinuria, thrombosis and bleeding. Six

gastrointestinal perforations occurred in patients receiving IFL plus bevacizumab. The results of this study demonstrated a significant improved activity and efficacy of the combination of bevacizumab with IFL in comparison to chemotherapy alone with manageable toxicities.

In the NO16966 study[44] about 1400 mCRC patients were randomized to receive chemotherapy (FOLFOX or XELOX) plus bevacizumab or chemotherapy (FOLFOX or XELOX) plus placebo as first-line treatment. The primary end point of this phase III study was PFS: the addition of bevacizumab to oxaliplatin-based regimens significantly increased PFS in comparison to chemotherapy alone (9.4 months *vs.* 8.0 months; HR 0.83, 97.5%CI 0.72-0.95, p=0.0023). Median OS was 21.3 months in the bevacizumab group and 19.9 months in the placebo group: this difference did not reach statistical significance (HR 0.89, 97.5%CI 0.76-1.03, p=0.077). RR was also superimposable in the two groups. The magnitude of the effect of bevacizumab seemed relatively less impressive if compared with that reported by Hurwitz and colleagues. As specified by the authors and suggested by Giantonio at the 2007 ASCO Annual Meeting[45], the main explanation could be the frequent discontinuation of bevacizumab plus chemotherapy before disease progression (in 71% of patients), which seems not to be related to unexpected adverse events. In fact, the toxicity profile of bevacizumab was in line with the data obtained in previous trials: the incidence of grade 3-4 thromboembolic events, hypertension and bleeding was 10%, 4% and 2% respectively, while grade 3-4 gastrointestinal perforations, proteinuria and wound healing complications were rare (<1%). Treatment discontinuation because of adverse events was reported in 30% of patients receiving bevacizumab.

The importance of maintaining bevacizumab until disease progression even if chemotherapy is stopped has been recently underlined by the MACRO trial[46]. Investigators randomized 480 patients to receive 6 cycles of XELOX plus bevacizumab followed by the same regimen or bevacizumab alone until disease progression. The trial suggested that bevacizumab could be non inferior to maintenance XELOX plus bevacizumab in terms of PFS (primary end point), since a detriment larger than 3 weeks with bevacizumab alone can be excluded, even though non-inferiority was not formally demonstrated (median PFS: 11.0 *vs.* 10.3 months, HR 1.07, 95%CI 0.84-1.36; upper non inferiority limit according to study design: 1.32). As reported by the studies evaluating optimal duration of chemotherapy alone, even in the MACRO trial patients in the single-agent bevacizumab arm experienced less grade 3-4 neurotoxicity and hand-and-foot syndrome.

The G.O.N.O. group conducted a phase II trial[47] in order to evaluate the combination of bevacizumab with the FOLFOXIRI regimen repeated every 2 weeks, for a total of 12 cycles, followed by a maintenance treatment with bevacizumab with or without 5-FU/LV. A total of 57 unresectable mCRC patients were enrolled. Objective response was obtained in 77% of patients and radical surgery of metastases was performed in 26% of patients (43% in patients with liver-only metastases). After a median follow-up of 18.4 months, median PFS was 13.4 months. The most common grade 3-4 bevacizumab-related toxicities were deep venous thrombosis (5%) and hypertension (11%). Results of this study are very promising and suggest that bevacizumab can be safely combined with the G.O.N.O. FOLFOXIRI regimen with manageable toxicities: in order to confirm these preliminary results and compare a triplet regimen plus bevacizumab with a standard doublet chemotherapy

(FOLFIRI) plus the same antiangiogenic agent, a phase III trial is currently ongoing and has recently completed accrual.

1.1.3. Role of anti-EGFR agents in metastatic colorectal cancer

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with an intracellular tyrosine kinase domain. Binding of specific ligands, such as epidermal growth factor (EGF) or transforming growth factor alpha (TGF-α), to the receptor causes the dimerization of single-chain EGFR and subsequent activation of receptor autophosphorylation through tyrosine kinase activity[48]. These molecular events initiate a cascade of intracellular signalling pathways, which ultimately regulate cancer cell proliferation and differentiation, apoptosis and survival, invasion and metastatic potential and tumor-induced neovascularisation.

Considering that deregulation of EGFR-controlled pathways is a common phenomenon in human epithelial carcinogenesis, EGFR was the first growth factor receptor to be proposed as a target for cancer therapy. Up today, two different classes of EGFR-inhibitors have been developed and successfully tested in clinical trials for malignancies of different origin: anti-EGFR monoclonal antibodies and small-molecule EGFR tyrosine kinase inhibitors[49].

Two anti-EGFR monoclonal antibodies are currently approved for the treatment of mCRC: cetuximab and panitumumab. Cetuximab is a chimeric IgG1 monoclonal antibody that competitively inhibits endogenous EGF/TGF-α binding targeting the EGFR extracellular domain with a consequent inhibition of cancer cell

proliferation and induction of apoptosis. Panitumumab is a fully humanized IgG2 monoclonal antibody that elicits a minimal immunogenic response, thus reducing the risk of hypersensitivity reactions. However, IgG2 antibodies are not able to induce antibody-dependent cell-mediated cytotoxicity (ADCC): the clinical impact of ADCC is not fully understood, but it is thought to contribute to the antitumor activity of IgG1 antibodies[49].

Recently, deeper insights into EGFR biology have led to the identification of *KRAS* mutational status as a key determinant of sensitivity to anti-EGFR treatment in mCRC[50] (see paragraph 1.2. for details). In fact, it is now well established that benefit from both cetuximab and panitumumab is limited to the *KRAS* wild-type population, while *KRAS* mutant tumors are resistant to anti-EGFR agents. This is particularly true for the most common mutations at codons 12 and 13, while for less frequent mutations the available evidence is less conclusive.

Several trials established the role of anti-EGFR agents in different lines of treatment in mCRC. The CRYSTAL study[51,52], a randomized phase III study evaluating FOLFIRI with or without cetuximab, demonstrated significant improvement for the experimental arm in RR (57.3% *vs.* 39.7%; p<0.0001), PFS (9.9 *vs.* 8.4 months; HR 0.696, 95%CI 0.558-0.867, p=0.0012) and OS (23.5 *vs.* 20.0 months; HR 0.796, 95%CI 0.670-0.946, p=0.0093) among *KRAS* wild-type patients. Interestingly, among patients with liver-limited disease RR increased from 44.4% to 70.6% with the addition of cetuximab to FOLFIRI. As regards secondary resection rate[53], cetuximab had a positive impact in patients unselected for *KRAS* mutations, since 7.0% of patients in the anti-EGFR arm were resected compared to 3.7% with FOLFIRI alone and R0 resection rates were 4.8% and 1.7%, respectively (p=0.002).

Among *KRAS* wild-type patients, radical surgery was achieved in 5.1% of patients treated with cetuximab compared with 2.1% of patients treated with chemotherapy alone (p=0.027), and for patients with liver-only metastases the percentages raise to 13.2% and 5.6%, respectively (p=0.15). The toxicity profile of the combination treatment, cetuximab plus FOLFIRI, was in line with that expected: the incidence of grade 3 skin reactions, mainly acne-like rash, was significantly higher in patients receiving the anti-EGFR antibody in comparison with those receiving FOLFIRI alone (skin reactions: 19.7% *vs.* 0.2%, p<0.001; acne-like rash: 16.2% *vs.* 0.0%; p<0.001). None of the skin-related toxicities reported were grade 4 and, in the cetuximab-treated group, grade of rash was shown to be associated with PFS. Also the incidence of grade 3-4 diarrhoea (15.7% *vs.* 10.5%; p=0.008) and infusion-related reactions (2.5% *vs.* 0.0%; p<0.001) was significantly increased in the cetuximab-FOLFIRI group. However, these toxicities were manageable and the combination treatment appeared feasible.

With regard to oxaliplatin-based chemotherapy, the most interesting results are those reported by the investigators of the randomized phase II OPUS trial[54,55]: among the 337 patients enrolled, there was a trend in favour of the cetuximab-containing arm compared to the FOLFOX-4 arm in terms of RR (46% *vs.* 36%; p=0.064), with super-imposable outcome in terms of PFS (median: 7.2 *vs.* 7.2 months; HR 0.931, 95%CI 0.705-1.230, p=0.62). When analysis was limited to the 179 patients with *KRAS* wild-type tumor, results achieved statistical significance in favour of cetuximab both in terms of RR (57% *vs.* 34%, p=0.0027) and PFS (median: 8.3 *vs.* 7.2 months; HR 0.567, 95%CI 0.375-0.856, p=0.0064). Interestingly, in contrast with the CRYSTAL trial, OPUS showed significantly poorer results in terms of activity

(RR: 34% *vs.* 53%, p=0.0290) and efficacy (median PFS: 5.5 *vs.* 8.6 months; HR 1.720, 95%CI 1.104-2.679, p=0.0153) for the cetuximab-containing arm among the 136 patients with a *KRAS* mutant tumor. Again, OPUS confirmed that a cetuximab-based treatment may be interesting in increasing the percentage of *KRAS* wild-type patients converted to resectability over chemotherapy alone (7.3% *vs.* 3.1%, p=0.22; in patients with liver-only metastases: 16% *vs.* 4.3%, p=0.35), even though data from such limited subgroups should be interpreted with caution.

Folprecht et al.[56] reported the results of the phase II CELIM trial, which randomized 111 patients with unresectable liver metastases to receive cetuximab with either FOLFOX-6 or FOLFIRI. In contrast with the unselected populations enrolled in CRYSTAL and OPUS, CELIM investigators excluded patients with extra-hepatic disease and precisely defined criteria for non-resectability (i.e. five or more liver metastases or liver metastases judged technically non-resectable by the local liver surgeon and radiologist on the basis of inadequate future liver remnant, infiltration of all hepatic liver veins, infiltration of both hepatic arteries or both portal vein branches). It should be noted that the trial did not enrol patients with potentially resectable liver metastases only: in fact, expected resectability was not considered an inclusion criterion and patients with extensive liver involvement were eligible. Resectability was re-assessed by a local multidisciplinary team using CT scans after 8 cycles of treatment and, if not feasible, every 2 months thereafter. As regards the primary endpoint, FOLFOX-6 and FOLFIRI achieved comparable RR in combination with cetuximab in the *KRAS* unselected population (68% *vs.* 57%, p=0.23), resulting in similar R0 resection rates (38% *vs*. 30%, respectively). A retrospective review by independent surgeons was made on the scans performed

baseline and after treatment by 68 patients and clearly indicates that resectability rates increased from 32% to 60% ($p<0.0001$) as a consequence of treatment activity.

Despite these intriguing results, treatment with anti-EGFR agents still has some dark sides that question the role of cetuximab and panitumumab in mCRC patients. The MRC COIN study[57], a large phase III trial conducted in the United Kingdom, randomized 2445 unselected mCRC into three different arms: continuous fluoropyrimidine (5-FU/LV or capecitabine) and oxaliplatin, intermittent fluoropyrimidine and oxaliplatin or continuous fluoropyrimidine and oxaliplatin plus cetuximab. The trial tried to answer two different questions, i.e. whether intermittent chemotherapy is non-inferior in terms of OS to continuous chemotherapy and whether the addition of cetuximab improves OS compared to chemotherapy alone. As regards the latter question, among the 729 *KRAS* wild-type patients investigators failed to demonstrate a significant difference in favour of the cetuximab-containing arm compared to chemotherapy alone in terms of OS (median: 17.0 *vs.*19.9 months; HR 1.04, 95%CI 0.90-1.20; p=0.68) and PFS (median: 8.6 *vs.* 8.6 months; HR 0.959, 95%CI 0.84-1.09; p=0.60), despite a marginally significant improvement in RR was proved (64% *vs.* 57%, p=0.049). Interestingly, a test for interaction suggested a differential effect for cetuximab on PFS according to the fluoropyrimidine administered (p=0.10), indicating a potential benefit in patients receiving 5-FU/LV and a detrimental effect with capecitabine: these data are confirmed by the authors by pooling the data of first-line trials with cetuximab both for PFS and OS. These results could possibly reflect the higher toxicity registered in the cetuximab arm when combined with XELOX, particularly in terms of severe mucosal injury (mainly grade 3 or more diarrhoea: 15% *vs.* 26%, p=0.0001) that led to a reduction in the

dose of capecitabine during the study. Therefore, even in molecularly selected patients the addition of cetuximab to an oxaliplatin-based chemotherapy in a large trial failed to demonstrate significant advantage, with the exception of a marginal improvement in RR. As regards secondary resection of metastases, no increase in potentially curative liver surgery was identified, with resection rates among *KRAS* wild-type patients who had liver-only metastases at baseline of 13% (12 out of 91 patients) in the control group and 15% (13 out of 87 patients) in the cetuximab group (p=0.74).

In line with these results are those from the NORDIC VII trial[58], which randomized 566 unselected mCRC patients to first-line therapy with a combination of bolus 5-FU and oxaliplatin (FLOX regimen), FLOX plus cetuximab until disease progression, or FLOX intermittently plus continuous cetuximab. The three arms achieved similar OS results, with no differences between continuous chemotherapy with or without cetuximab (19.7 *vs.* 20.4 months; HR 1.06; 95%CI, 0.83-1.35; p=0.67). Surprisingly, even among *KRAS* wild-type patients OS did not differ between these arms (20.1 *vs.* 22.0 months; HR 1.14, 95%CI 0.80-1.61, p=0.66), as did PFS (7.9 *vs.* 8.7 months, HR 1.07, 95%CI 0.79-1.45, p=0.66). It should be underlined however that sample dimension in NORDIC trial was not adequately powered for subgroup analysis. Anyway, NORDIC results stress the need for optimal companion chemotherapy with anti-EGFR agents in mCRC, and bolus 5-FU such as in the FLOX regimen is probably a not preferable schedule for combination with oxaliplatin.

As previously discussed, triplet chemotherapy plus anti-EGFR may be a promising approach to maximize the activity of first-line treatment and possibly

increase the conversion to resectability rate. Up to now, data from phase II trials with different chemotherapy schedules are available. Garufi et al.[59] recently reported the results of a triplet regimen with chrono-modulated irinotecan, 5-FU/LV and oxaliplatin (named chrono-IFLO) administered as neoadjuvant chemotherapy to increase the resectability of colorectal liver metastases in 43 initially unresectable mCRC patients due to extensive liver involvement or presence of extra-hepatic disease. Patients were not selected for *KRAS* status, but mutations were retrospectively evaluated in 37 out of 43 patients: 81% of analyzed samples were *KRAS* wild-type. The chrono-IFLO plus cetuximab combination achieved an interesting 79.1% RR, allowing radical resection of metastases in 26 patients (60%) after a median of 6 cycles before surgery, suggesting that a rapid shrinkage with a very active combination may shorten the duration of preoperative chemotherapy.

A possible concern with the combination of triplet chemotherapy and anti-EGFR agents is represented by the increased risk of mucosal toxicity: the POCHER trial reported a high rate of grade 3-4 diarrhoea (93% of patients), requiring dose reduction for all the cytotoxic agents. After dose reduction, incidence of diarrhoea decreased, but still occurred as grade 3-4 in 36% of treated patients, with an additional 26% of patients experiencing grade 2 diarrhoea: these results suggest that chrono-IFLO plus cetuximab is feasible, but requires adequate patient selection to avoid unacceptable toxicity.

A French group[60] tested cetuximab in combination with a more suitable triplet schedule for outpatient management (FOLFIRINOX) in a phase II trial in clinically and molecularly unselected mCRC patients aged 75 years or less and with good performance status, with complete RR as the primary end point. Preliminary

interesting results have been presented: among 22 evaluable patients, 4 complete and 18 partial responses were reported (RR, 82%). Gastrointestinal tolerability was greater than that reported by POCHER investigators: however, grade 3-4 diarrhoea and neutropenia (even despite primary G-CSF prophylaxis) still occurred in 35% and 28% of the patients, respectively. Taken together, these two trials confirm that combining cetuximab with a three-drug regimen may result in consistent increase in activity, but efforts should be made in order to improve the feasibility of these regimens and better select patients on the basis of optimal staging techniques and molecular characterization.

As regards panitumumab, final results of the phase III PRIME trial[61] have been recently published. PRIME randomized 1183 unselected mCRC patients to first-line FOLFOX-4 with or without panitumumab and results of the study were prospectively analyzed by tumor *KRAS* status. In the wild-type *KRAS* population, adding panitumumab to chemotherapy significantly improved PFS (primary endpoint) compared with chemotherapy alone (median, 9.6 *vs.* 8.0 months; HR=0.80, 95%CI 0.66-0.97, p=0.02), while a non-significant increase in OS was observed (median OS, 23.9 *vs.* 19.7 months; HR=0.83, 95% CI 0.67-1.02, p=0.072). As regards RR, despite a strong trend the increase in activity by the addition of panitumumab to chemotherapy did not reach statistical significance (55% *vs.* 48%; p=0.068). As a result, panitumumab plus FOLFOX-4 did not improve secondary resection rate over FOLFOX-4 alone: in fact, metastasectomy of any site was attempted in 10.5% of patients treated with the anti-EGFR agent and in 9.4% of patients treated with chemotherapy alone, while R0 resection was achieved in 8.3% and 7.0% of patients, respectively. As in other series, the addition of the anti-EGFR

antibody to oxaliplatin-based chemotherapy resulted in worse outcome in terms of PFS (median, 7.*3 vs.* 8.8 months; HR 1.29, 95%CI 1.04-1.62, p=0.02) and OS (median, 15.5 *vs.* 19.3 months; HR 1.24, 95%CI 0.98-1.57, p=0.068) among *KRAS* mutant patients.

Globally considered, first-line trials testing anti-EGFR antibodies in mCRC demonstrate that the combination of such agents with chemotherapy may improve activity and, to a lesser extent and with less consistent results across trials, increase the efficacy of chemotherapy. If *KRAS* status evaluation is recognized as an essential element for treatment allocation, molecular refinement of patient selection may help to further improve the results of anti-EGFR agents. Moreover, companion chemotherapy is probably more relevant in order to maximize the benefit from these agents when compared to bevacizumab, as confirmed by COIN, PRIME and NORDIC trials which suggest oxaliplatin-based doublets as a not preferable alternative to irinotecan-containing ones. If the results of the OPUS and CELIM trials partially reassure about the possibility of adding an anti-EGFR antibody to an infusional 5-FU/LV and oxaliplatin (FOLFOX) regimen, results presented by the COIN and NORDIC investigators indicate that oxaliplatin plus either oral fluoropyrimidine (XELOX) or bolus 5-FU/LV (FLOX) should be avoided when patients are considered for a first-line cetuximab-containing combination.

1.2. Prognostic and predictive factors in metastatic colorectal cancer

Together with improvements in the medical management of mCRC, deeper insights into the molecular basis of colorectal carcinogenesis have provided additional targets for new drug development and potential (or validated) predictive and prognostic biomarkers[62,63]. Despite these advances, however, our ability to predict disease course and patient response to treatments is relatively poor, and new hypothesis are being tested in order to optimize treatment decision in single cases.

A comprehensive description of all the molecular variables evaluated as prognostic and predictive factors in mCRC is beyond the scope of this Introduction: we will therefore focus on the already validated biomarkers which have entered routine clinical practice and on the most promising alternatives which are still passing through the hard challenge of validation.

The relevance of *KRAS* mutational status evaluation in mCRC patient candidate to anti-EGFR treatment has been already mentioned in the previous paragraph. Since the evaluation of EGFR expression by immunohistochemistry was not demonstrated as a useful tool to predict treatment efficacy[64,65], many efforts have been made to identify potential predictors of benefit from anti-EGFR monoclonal antibodies. Attention has been therefore focused on intracellular mediators involved in the transduction of EGFR signal and both the KRAS/BRAF/MAPKs and the PTEN/PI3K/pAKT pathways have been investigated[66].

KRAS belongs to the *RAS* family of genes (comprising *KRAS*, *NRAS*, and *HRAS*) that encode guanosine-5´-triphosphate (GTP)-binding proteins. *KRAS* mainly acts as an effector of the ligand-bound EGFR through *BRAF* and the *MAPK* axis, but

can also activate PI3K through direct interaction with its catalytic subunit[67]. About 32-40% of CRC cases harbour a *KRAS* mutation: most (85-90%) of these mutations occur in codons 12 or 13, with the remaining mainly occurring in codons 61 (5%) and 146 (5%)[68]. These mutations abolish the intrinsic GTPase activity of Ras protein, leading to the constitutive activation of the RAS/RAF/MAPKs cascade[69]: signalling events are thus independent from EGFR control, since KRAS accumulates in the active GTP-bound conformation.

Several retrospective experiences[70,71,72,73,74], then corroborated by the results of *post-hoc* analyses of large phase III randomized studies[52,75,76], have evidenced the role of *KRAS* codon 12-13 activating mutations as predictors of resistance to anti-EGFR antibodies. The abovementioned results of first-line trials are confirmed by the analysis of phase III trials that randomized heavily pretreated mCRC patients to anti-EGFR monotherapy or BSC alone: notably, results of these trials are not affected by the potential confounding effect of the associated chemotherapy regimens. When compared to BSC, both cetuximab and panitumumab demonstrated a survival benefit only for patients with *KRAS* wild-type tumors. No responders were identified among patients with *KRAS* mutated disease treated with panitumumab in comparison with the 17% of patients with *KRAS* wild-type tumors. Similar findings were reported in terms of PFS: the treatment effect in the *KRAS* wild-type (HR 0.45, 95%CI 0.34-0.59) was significantly greater ($p<0.0001$) than in the mutant group (HR 0.99, 95%CI 0.73-1.36)[76]. On the basis of these results, panitumumab was initially approved by regulatory authorities for the treatment of mCRC patients with *KRAS* wild-type disease[77]. Analogous results were obtained by the analysis of *KRAS* mutational status in samples from patients enrolled in the CO.17 trial that

randomized fluoropyrimidine-, irinotecan- and oxaliplatin-refractory mCRC patients to cetuximab or BSC. The anti-EGFR antibody significantly improved PFS (3.7 months *vs*. 1.9 months, HR 0.40, p<0.001) and OS (9.5 months *vs.* 4.8 months, HR 0.55, p<0.001) only among patients with *KRAS* wild-type tumors[75]. As a result of the above reported results, demonstrating the negative predictive value of *KRAS* codon 12 and 13 mutations, the use of cetuximab is now restricted to patients with *KRAS* wild-type disease[78].

Unfortunately, although the specificity of *KRAS* testing as predictor of resistance to anti-EGFR monoclonal antibodies is quite high, the sensitivity is less satisfactory[79], so that while patients bearing such alterations do not benefit from treatment, a percentage of patients with *KRAS* wild-type status does not achieve benefit from anti-EGFR antibodies. Additional predictive biomarkers are therefore eagerly awaited in order to refine molecular selection for anti-EGFR treatment allocation.

KRAS activating mutations, occurring in codons other than 12 and 13, have been described in mCRC. Codon 61 and 146 mutations[80] determine the constitutive activation of RAS protein, by reducing its intrinsic GTPase activity or increasing its affinity for GTP. It has been recently reported that, among 87 patients with *KRAS* codon 12 and 13 wild-type disease, none of the patients bearing codon 61 or 146 mutations responded to cetuximab plus irinotecan, compared to 22 out of 68 wild-type patients (p=0.096) and *KRAS* rarer mutations were also associated with shorter PFS (HR 0.46, p=0.028)[81]. More recently, a European retrospective study[82] over 1022 samples of cetuximab-treated patients showed that codon 61 mutations (but not codon 146 mutations) had an adverse effect similar to codon 12 mutations: in fact,

patients harbouring codon 61 mutant tumors had a significantly lower RR than did patients with wild-type disease (0% *vs.* 35.7%, p=0.0055), but this difference was not observed among patients with codon 146 mutant tumors (18.2% *vs.* 36.9%, p=0.34). Moreover, authors found that codon 146 mutations co-occurred with other *KRAS* mutations, suggesting that this might not be an important oncogenic site. Moreover, in a large retrospective pooled exploratory analysis of chemotherapy-refractory patients[83], a positive association between *KRAS* G13D mutations and cetuximab treatment was seen in regard to better OS and PFS: compared to patients with other *KRAS*-mutated tumors, the 32 patients with G13D-mutated tumors treated with cetuximab had longer median OS (7.6 *vs.* 5.7 months; HR 0.50, 95%CI 0.31-0.81, p=0.005) and longer median PFS (4.0 *vs.* 1.9 months; HR 0.51, 95%CI 0.32-0.81, p=0.004). There was a significant interaction between *KRAS* mutation status (G13D *vs.* other *KRAS* mutations) and OS benefit with cetuximab treatment (HR 0.30, 95%CI 0.14-0.67, p=0.003). Tejpar et al. have recently presented data regarding G13D mutation for first-line OPUS and CRYSTAL trials: heterogeneous treatment effects were seen for all endpoints across the mutation types with significant treatment interaction by *KRAS* mutation status for RR, PFS and OS and among the 83 patients harbouring a G13D mutant tumor the addition of cetuximab improved the activity and the efficacy of chemotherapy, even though to a lesser extent, compared to wild-type patients[84]. However, other confirmatory results from prospective randomised trials are needed before definitive conclusions about the predictive role of the G13D mutation can be inferred.

Among the other molecular determinants with already validated usefulness in the clinics is *BRAF* mutational status. *BRAF* is a member of the *RAF* gene family

(comprising *BRAF*, *ARAF1* and *RAF1*) which encodes a serine-threonine protein kinase that is a downstream effector of activated *KRAS*. The most frequent (over 95% of the cases) *BRAF* mutation is the V600E mutation within the kinase activation domain of the B-RAF protein[82]. Functional consequences of the V600E mutation are not completely understood, but it is conceivable that it results in increased MAPK1/3 activation, as seen for mutant *KRAS*, since BRAF acts downstream of KRAS to activate MAP2K. *BRAF* mutations occur in about 10-15% of CRC cases[85], with higher percentages reported in earlier lines of therapy. Interestingly, *KRAS* and *BRAF* mutations are mutually exclusive in CRC[82], suggesting that they could identify different tumor subtypes: in fact, *BRAF* mutant tumors are clinically and histologically different from *KRAS*-mutant ones[86]. Moreover, from a biological perspective *BRAF* mutations are associated with the CpG island methylator phenotype (CIMP) and microsatellite instability, whereas *KRAS* mutations are more common in CIMP-low and microsatellite-stable tumors[86]. Nowadays V600E-mutant *BRAF* is recognized as probably the most important negative prognostic determinant in mCRC, while the prognostic role of *KRAS* mutations (beyond their proved predictive effect for anti-EGFR agents) has not been convincingly demonstrated[85]. All retrospective and *post-hoc* analyses of *BRAF* mutations in mCRC series clearly showed that the prognosis of mutant tumor is worse than that of wild-type cases. Even in the first-line setting, the CRYSTAL trial reported poor PFS and OS results among patients with *BRAF* mutant tumor both in the control (5.6 and 10.3 months, respectively) and experimental arm (8.0 and 14.1 months, respectively)[52].

On the other hand, great debate is ongoing about the additional predictive role of *BRAF* V600E mutation in *KRAS* wild-type mCRC patients receiving anti-EGFR

agents. In most studies, no response was seen with the use of cetuximab or panitumumab in patients with *BRAF* mutant mCRC in the chemotherapy refractory setting[81,82,87,88,89] and the available data strongly suggest that this mutation confers resistance to anti-EGFR monoclonal antibodies. Among previously untreated patients enrolled onto the CRYSTAL study, the results are limited but the small sample size prevents any conclusion: however, no clear benefit in terms of RR (15.2% *vs.* 19.2%, p=0.91) and only a modest gain in efficacy (median PFS: 5.6 *vs.* 8.0 months; HR 0.934, 95%CI 0.425-2.056, p=0.87) from the addition of cetuximab to chemotherapy were evident at subgroup analysis[52].

CHAPTER 2. *CANCER STEM CELLS: ESSENTIALS OF BIOLOGY*

1.1. Cancer stem cells and colorectal cancer

The classical model of cancer development and progression (i.e. the *stochastic model*) holds that most or all cancer cells have inherent tumorigenic potential and have the same possibility to develop oncogenic mutations: in such a model, tumor heterogeneity is caused by subclones of tumor cells that result from a combination of different microenvironments and random genetic changes[90].

In the last few years an alternative approach to cancer modelling (the so called *cancer stem cell [CSC] model*) has been proposed and is now supported by growing evidences[90]. Stem cells are defined as cells that have two key properties, i.e. the ability to perpetuate themselves (or *self-renewal*) and to generate all the differentiated cells of the tissue of origin through differentiation (*multipotency*)[91]. Self-renewal is the result of asymmetric division, which generates a quiescent stem cell (with extensive proliferative capacity and the same developmental potential as its parent) and a committed progenitor. Normal stem cells and CSCs share similar signalling pathways (such as Wnt, Sonic Hedgehog and Notch) and epigenetic modulators (such as Polycomb genes) for regulation of self-renewal[92]. According to the CSC model, human malignancies are hierarchically organized, with CSCs at the apex, because they are the only cancer-initiating cells within a tumor. CSCs display three essential characteristics[93]: 1) the expression of a repertoire of markers common

to stem and progenitor cells; 2) unlimited growth *in vitro* using media optimized for normal stem cell cultures; 3) ability to reproduce the parental tumor upon injection in immunocompromised mice. Through asymmetric division, CSCs generate also non-self-renewing cells that finally give rise to tumor heterogeneity. Importantly, the stochastic model and the CSC model should not be seen as mutually exclusive[90]. In fact, CSCs (as other malignant cells) likely exhibit genomic instability and in the course of tumor progression different subclones of CSCs may develop: these distinct subsets may then give rise to genomically distinct non-tumorigenic cells. Intriguingly, CSCs may arise from normal tissue stem cells, but recent studies suggest that they can also develop from progenitor cells, i.e. non self-renewing cells that acquire the capacity for self-renewal[90].

Models of carcinogenesis have not only theoretical implications, but also relevant practical significance. According to the stochastic model, effective treatment of cancer must involve eradication of all cancer cells, since all cancer cells are tumorigenic. On the contrary, if we interpret cancer as a CSC-driven disease, effective treatment modalities will be based on direct targeting of CSCs, rather than non-tumorigenic cells, which can not further sustain cancer growth[90]. Moreover, since CSCs represent only a small fraction of the tumor mass, it is conceivable that objective response, as evaluated by conventional radiological imaging may be not the best way to evaluate strategies targeting the CSC compartment[94]. Finally, the CSC model prompts us to rethink the way we investigate the mechanisms of resistance to anticancer treatments and we identify potential predictive factors through pharmacogenomic analyses: indeed one possible pitfall of these studies could reside in the underestimation of the contribution of CSCs to drug response[95].

Even though the CSC model may not apply to all cancer types, recent literature data provide reliable evidence that cells with stem cell properties may be identified in most of solid malignancies[90,92]. As regards CRC, convincing experimental evidence supporting the existence of colonic CSCs has been recently provided[96,97]. Several authors have independently demonstrated that the CRC tumorigenic cell population can be isolated by means of the expression of different cell surface biomarkers: such experiments used flow cytometric analysis and spheroid culture formation to identify cells through specific surface markers that might enrich for the tumorigenic stem cell compartment of the tumor mass. Most of these studies used CD133 to identify a colon cancer-initiating cell population in human tumors[98,99], but a plethora of putative CSC markers have been proposed (**Table 2.1**). CD133, also known as prominin-1, is structured as a five transmembrane domain molecule and is located in the apical plasma membrane protrusions of embryonic epithelial structures[100]: despite some intriguing hypothesis suggesting a role in the regulation of plasma membrane structure and function, the exact role of CD133 remains unknown. $CD133^+$ cells account for approximately 2.5% of the bulk tumor cells and do not express the cytokeratin (CK) 20 epithelial marker (which identifies terminally differentiated cells), while they express the epithelial adhesion molecule BerEp4 (also known as EpCAM)[98,99]. In clonogenic assays, cultured $CD133^+$ cells are capable of colonies and crypt-like structures formation. When cultured in serum-free liquid medium, they organize in spheres which can be transplanted into immunosuppressed non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice resulting in tumor formation, whereas the $CD133^-$ cell population was unable to generate tumors[98,99]. Some authors calculated that the frequency of

CRC-initiating cells in an unfractionated population of cancer cells is approximately one out of 5.7×10^4: however, when enriched for $CD133^+$ cells, frequency rises up to one out of 262 cells[96]. Further supporting the existence of a real CSC population in CRC, tumor xenografts generated by $CD133^+$ cells have the same morphologic features of the parental tumor (with $CD133^+$ and $CD133^-$ cells present at similar ratios to the original tumor) and can be maintained upon serial transplantation. Since CD133 is expressed at lower frequency also in the normal colonic tissue, it can be hypothesized that CSCs in CRC arise from malignant transformation of normal colonic stem cells[96,97]. Some argued against the role of CD133 as optimal marker for CSC identification, since CD133 mRNA and protein are expressed by both differentiated cancer cells and CSCs in CRC specimens[101]. Methodological issues should however be taken into account, since it has been recently shown that only one CD133 epitope (AC133) is specifically expressed by CSCs due to differential glycosilation of the protein[102].

Alternatively, other groups employed additional (or alternative) markers for CSCs isolation from CRC samples. Dalerba et al.[103] proved that, as previously seen for $CD133^+$ cells, cells expressing both CD44 (which acts as a receptor for the extracellular matrix component hyaluronan, thus regulating cell survival, motility and chemoresistance) and EpCAM (i.e. $CD44^+/EpCAM^{high}$) resulted in the generation of tumor xenograft with high frequency when injected subcutaneously into NOD/SCID mice, whereas $CD44^-/EpCAM^{low}$ cells lack tumor-initiating activity. Further subfractionation of the $CD44^+/EpCAM^{high}$ cell population has been attempted by using the mesenchymal stem cell marker CD166: the tumorigenic potential of the $CD44^+/EpCAM^{high}/CD166^+$ (as measured by xenograft formation)

increased, suggesting that a combination of markers might be a more reliable indication of stemness than CD133 alone. However, even with the use of these apparently more robust markers uncertainties exist, since CD44 expression may be demonstrated by immunohistochemistry not only in the stem cell compartment at the crypt bottom but also in cells within the proliferative compartment of normal colonic epithelium[96].

More recently, aldehyde dehydrogenase 1 (ALDH1) has been proposed as a promising new marker for both normal and malignant human colonic stem cells[104]. Flow cytometric isolation of cancer cells based on enzymatic activity of ALDH1 and subsequent injection into NOD/SCID mice not only resulted in the formation xenograft tumors, but also proved highly effective in generating cancer (since as few as 25 cells were sufficient) in animals. Interestingly, further refinement of CSC isolation by the use of a second marker ($CD44^+$ or $CD133^+$ serially) only modestly increased enrichment based on tumor-initiating ability. Thus, in the experience by Huang et al. ALDH1 seems to be a specific marker for identifying, isolating, and tracking human colonic stem cells during CRC development.

1.2. Preliminary clinical implications of the cancer stem cell hypothesis in colorectal cancer

Intriguing despite somewhat conflicting results in colorectal CSC isolation have prompted the way toward the investigation of clinical implications of CSC markers in patient with CRC. Results are still limited, but available preliminary

experiences are moving faster the translation of CSC concepts in the medical practice.

Horst and colleagues[105] first evaluated CD133 expression by immunohistochemistry in 77 CRC specimens selected as moderately differentiated T2 or T3 N0 M0 tumors. As previously shown, author confirmed that the CD133 antigen is localised on the glandular-luminal surface of CRC cells, expression being confined to the apical luminal surface of CRC cells with glandular differentiation, whereas undifferentiated tumor cells at the front of invasion (the so called tumor "buds") are generally $CD133^{-}$. Moreover, in this series CD133 levels were independently associated with prognosis: in fact, $CD133^{high}$ tumors (i.e. showing more than 50% positive glands) were associated with a significantly lower 5-year and 10-year OS compared to tumors showing $CD133^{low}$ expression. In line with these data, Li et al.[106] reported a lower 5-year OS survival rate among patients with a higher percentage of $CD133^{+}$ cells (≥5%) than in those with a lower percentage of $CD133^{+}$ cells in a series of 104 stage IIIB CRC cases. Artells et al.[107] assessed CD133 expression measuring mRNA levels by real-time quantitative polymerase chain reaction (RT-PCR) in tumor and matched normal tissue from 64 CRC patients with radically resected stage I–III disease, and correlated tumor CD133 levels with clinical and pathological characteristics and clinical outcome. Among the 60 patients with detectable CD133 mRNA, expression levels were significantly higher in tumor than in normal tissue and higher levels of CD133 expression were associated with shorter relapse-free interval (RFI) and OS, and prognostic significance of CD133 levels was retained also at multivariate analyses. Similar association with risk of

recurrence were achieved measuring CD133 mRNA levels in peripheral blood samples of another series of 66 colon cancer patients with stage I-IV disease[108].

As previously discussed in this chapter, CD133 may be not the optimal markers for colorectal CSC isolation. Interestingly, Saigusa et al.[109] evaluated OCT4 and SOX2 (which are putative markers of stem cells) together with CD133 by RT-PCR and immunohistochemistry among 33 patients with rectal cancer undergoing preoperative chemoradiation and found that higher levels of the three studied genes were associated with poorer disease-free survival (DFS). Lugli et al.[110] reported the results of a tissue microarray of 1420 primary CRC cases and 57 normal mucosa samples analyzed by immunohistochemistry for protein markers CD133, CD44, CD166, EpCAM, and ALDH1: differences between normal tissue and cancer were observed for all markers and, intriguingly, loss of membranous CD166 and CD44 was associated with higher pathological T and N stage, an infiltrating growth pattern and worse OS in univariate analysis only. Due to their role as adhesion molecules, authors tested the hypothesis that CD44 and CD166 loss might result in higher invasive potential of tumor cells *in vitro*: for all the three cell lines evaluated, $CD44^{-}/CD166^{-}$ cells exhibited significantly higher invasive potential than the $CD44^{+}/CD166^{+}$ counterparts.

Probably, the most interesting study evaluating the prognostic value of CSC markers among colon cancer patients is the one recently published by Iinuma and colleagues[111]. Authors measured by RT-PCR the expression of CEA, CK19, CK20, and CD133 mRNA from blood samples (collected before surgery) of 735 CRC patients: after initial testing in a 420 patient-cohort, validation was achieved in the remaining 315 cases. The expression of CEA/CK/CD133 was associated with

significantly poorer DFS and OS in Dukes' B and C stage CRC cases, and this prognostic value was retained also at multivariate analysis.

As seen for CSC markers, no consensus exist about the optimal methods for CSC detection in the real clinical scenario: therefore, results of these preliminary experiences are limited by differences in the techniques used for CSC detection and cut-off values for patient stratification[112,113]. Moreover, data interpretation is made difficult by the small sample size of the published series, which prevents any definitive conclusion about the usefulness of CSC markers in the clinics[112]. Future studies exploring different methods on larger series will help to clarify definitively the prognostic implications of colorectal CSCs.

1.3. Epigenetics and cancer stem cells: role of Polycomb Repressor Complexes

The study of epigenetic (i.e. DNA sequence-external) modifications has recently gained increasing importance in the study of cancer biology, being moved forward by the rapidly evolving field of CSCs in solid malignancies. Epigenetic alterations are represented by losses (or gains) of DNA methylation and deviant patterns of histone modifications: DNA methylation and histone modifications are recognized mechanisms that can influence DNA accessibility, which is essential for DNA repair, DNA replication, and gene transcription[114,115].

Since epigenetics is a key regulator of multiple cellular processes, including the balance between self-renewal and differentiation in both embryonic and adult stem cells, it is conceivable that epigenetic modifications play a crucial role in CSC

behaviour[92]. Moreover, recent evidences suggest that CSC mechanisms of resistance to cytotoxic agents may be at least in part based on epigenetic gene regulation of DNA repair, apoptosis and cell-cycle control[116], thus opening the way toward therapeutic reversal of epigenetic modifications as a potential resensitizing strategy to conventional agents.

In comparison to gene mutations, which have been classically recognized as the key event in cancer development, activation or inactivation of specific genomic regions through epigenetic chromatin state modifications confers plasticity to stem cells, an essential property for maintenance of tissue homeostasis through multipotency[117]. Moreover, if the contribution of genetic alterations to malignancy is well understood, a model of carcinogenesis based only on genetic abnormalities can not account for the potential deleterious effects of a high mutation rate (for example, the trigger of apoptosis in highly mutated cells)[118] or for the lack of identifiable mutations in classical progression-related genes in some tumors[119]. Feinberg et al. have recently proposed an epigenetic progenitor model of human cancer, in which CSCs might originate from early epigenetic events (or *epimutations*) occurring in normal tissue stem cells, transit-amplifying cells or bone marrow-derived cells[119]. Epigenetic modifications thus offer a useful instrument to explain tumor heterogeneity compared with the simplified model of sequential mutational events.

In line with this model, epigenetic modifiers have been proved essential for stem cell biology. Polycomb group (PcG) genes probably represent the most studied and well-known epigenetic effectors in CSCs. First discovered for its role in *Drosophila* development, PcG are a highly evolutionarily conserved gene family, which encode for a group of epigenetic silencers, mainly organized in two

functionally and biochemically distinct multiprotein complexes, called Polycomb repressive complexes (PRCs) 1 and 2 (PRC1 and PRC2)[120,121]. PRC2 is involved in gene silencing initiation, through methylation of histone H3 lysine 9 and 27, and histone H1 lysine 26, all of which are markers of inactive chromatin. PRC2 comprises several components, including the core components enhancer of zeste 1 and 2 (EZH1 and EZH2), suppressor of zeste-12 (SUZ12), and embryonic ectoderm development (EED). SUZ12 and EED are required for complex stability and for the methyltransferase activity of the EZH2, which mediate histone H3 lysine 27 trimethylation (denoted as H3K27me3)[122]. PRC1 binds to H3K27me3 and definitively complete gene silencing through ubiquitination of lysine 119 of histone H2A (denoted as H2AK119ub). As PCR2, also PCR1 consists of several proteins including CBX2 (chromobox homolog 2) or related homologs (responsible for binding to H3K27me3), RING1A (ring finger protein 1A) or RING1B (which catalyse ubiquitination) and BMI1 (B-cell-specific Moloney murine leukemia virus integration site 1) or PCGF2 (polycomb group ring finger 6/Mel18), which are involved in the regulation of the ubiquitination activity[120]. These epigenetic modifications regulate gene transcription by creating an obstacle to transcription factor and RNA polymerase binding and by recruiting DNA methyltransferases (DNMT) (DNMT1, DNMT3A and DNMT3B)[123,124].

BMI1 and EZH2 are the two main actors in normal stem cells and CSCs epigenetics. As regards BMI1, this central role in stem cell behaviour is mainly mediated through several key pathways, including anchorage-independent growth, Wnt and Notch signalling[95]. On the other hand, EZH2 is required for E-cadherin silencing, which represents an essential step in the development of an invasive

phenotype in epithelial tumors[125]. Focusing on the role of *EZH2* in cancer development and progression, initial evidences of a strict link between this key epigenetic effector and malignancy were derived from microarray studies in prostate and breast cancer, and were subsequently confirmed in other tumor types[126]: in these series, EZH2 overexpression has been generally associated with increased tumor aggressiveness, which appears in line with *in vitro* studies proving the oncogenic potential of EZH2 by induction of anchorage-independent colony growth and invasion[126]. Moreover, since an intimate link exist between DNMTs and EZH2, PRC2 might be involved in aberrant methylation of CpG islands at specific gene promoters, a distinctive feature frequently found in many cancers, as demonstrated by the concomitant presence of aberrant CpG island methylation and enrichment of PRC2 and H3K27 methylation[127,128].

EZH2 overexpression may be triggered by deletions of microRNA-101, which is a negative regulator of *EZH2* expression, as described in prostate cancer[129]. More recently, acquired *EZH2* mutations resulting in gain of function in EZH2 activity have been identified in haematological malignancies. In particular, different heterozygous mutations at amino acid Y641 within the SET domain have been found in 7% of follicular lymphomas and 22% of diffuse large cell B-cell lymphomas of germinal centre origin[130]. While normal EZH2 mediates the first methylation reaction of histone H3K27 more efficiently than the subsequent mono- to di- and di- to trimethylation reactions, the Y641 mutants display opposite properties[131]: thus, heterozygous Y461 mutants cooperate with wild-type EZH2 to increase levels of H3K27me3.

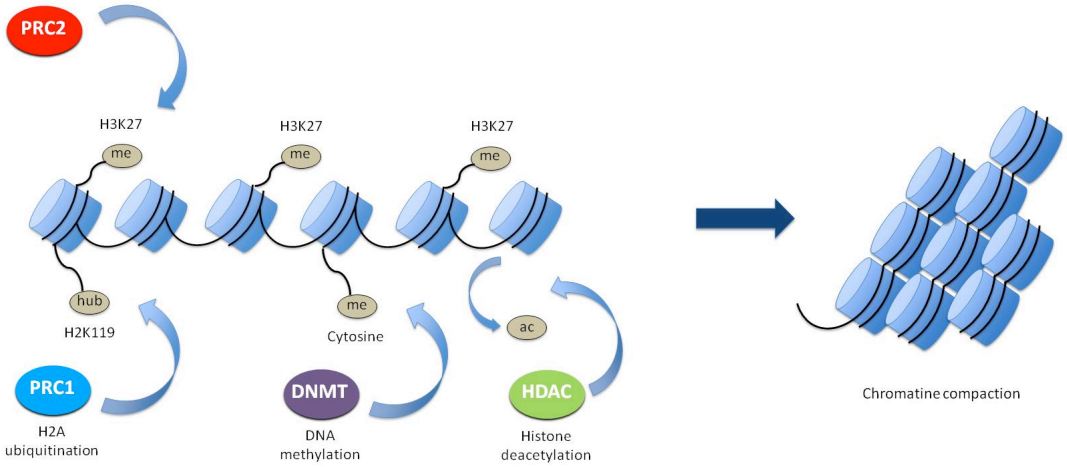
Intriguingly, EZH2 have been recently involved also in the regulation of tumor angiogenesis[132]. Lu et al. have recently published their results showing that *EZH2* expression in either tumor cells or tumor vasculature is associated with higher tumor stage and grade and is predictive of poor clinical outcome in a series of 180 epithelial ovarian cancers. Moreover, higher *EZH2* expression in the vasculature was associated with greater microvessel density. VEGF was shown to induce *EZH2* expression through a paracrine circuit: in turn, EZH2 methylates and silences *vasohibin1* (*vash1*), an antiangiogenic gene. *EZH2* silencing in the tumor-associated endothelial cells resulted in inhibition of angiogenesis through the increase in *vash1* transcription: this action reduced ovarian cancer growth, a result which is further enhanced when *EZH2* is silenced in tumor cells too. Therefore, *EZH2* may represent an intriguing target for novel anticancer agents not only for its direct effect on the regulation CSC behaviour, but also for its indirect effect on tumor growth through regulation of angiogenesis.

Table 2.1. Markers of normal intestinal stem cells and colorectal CSCs

	Marker	Function	Reference
Normal intestinal stem cells	Musashi-1	RNA-binding protein	96, 97
	Hes-1	Transcriptional repressor	96, 97
	EphB receptors	Cell surface receptors	96, 97
	BMI1	Policomb-repressor protein	96, 97
	Lgr5	Unknown, WNT target gene	96, 97
	Aldh1	Enzyme	96, 97
Colorectal CSCs	CD133	Unknown	98, 99
	CD44	Hyaluronic acid receptor	103
	CD166	Adhesion molecule	103
	Aldh-1	Enzyme	104

Abbreviations: CSCs, cancer stem cells.

Figure 2.1. Simplified view of PcG function



Polycomb repressive complex 2 (PRC2) catalyzes histone H3 lysine 27 (H3K27) trimethylation, resulting in the recruitment of Polycomb repressive complex 1 (PRC1), which in turns ubiquitinates lysine 119 of histone H2A (H2K119), DNA methyltransferase (DNMT) and histone deacetylase (HDAC): these changes finally lead to chromatin compaction and transcriptional repression [modified from ref. 126].

CHAPTER 3. *EZH2 POLYMORPHISMS AND OUTCOME OF METASTATIC COLORECTAL CANCER PATIENTS*

3.1. Rationale

As previously discussed, mCRC is one of the most frequent malignancies and one of the leading causes of cancer death in Western countries[1,2]. The therapeutic armamentarium against mCRC has rapidly increased in the last decade[4,7]: for the majority of mCRC patients, doublet chemotherapy with a fluoropyrimidine plus either irinotecan or oxaliplatin combined with a biologic agent is nowadays considered the preferred treatment option in first-line[7]. In particular bevacizumab, a fully humanized monoclonal antibody directed against the VEGF, proved to improve the efficacy of chemotherapy alone in several phase III trials with different regimens[43,44,45] and is approved in combination with fluoropyrimidine-based chemotherapy for the first- or second-line treatment of mCRC patients[a].

Despite these profound changes, long-term prognosis remains unfavourable, median OS not exceeding 24 months even in most recent studies[7]. Results from clinical trials and everyday practice clearly show that among mCRC patients, outcome widely vary according to several factors. Up to now, reliable prognostic parameters are represented by basal clinical and laboratory variables, validated

[a] http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/125085s01691bl.pdf

among thousands of mCRC patients often treated with older chemotherapy regimens[133].

With the few promising aforementioned exceptions[63], validated molecular markers of disease course are still lacking. In addition, genetic variants of drug targets or drug-metabolizing enzymes have been extensively tested as putative predictors of benefit from therapy: this has led to intriguing but often conflicting results among different series, making the aim of personalized medicine a challenge[134,135].

Recent evidence indicates that CRC is a stem cell-driven disease. Colorectal CSCs, mainly identified based on the CD133 surface marker expression, account for a minority (less than 3%) of the total tumor mass[98]. They share with normal stem cells the two key properties of stemness: the ability to self-renew and to differentiate into many cell types. In addition, colorectal CSCs display a unique tumorigenic potential[98], are more resistant to 5-fluorouracil-based chemotherapy[136] and more invasive than the other tumor cells[137]. For these reasons, CSCs are thought to be the seeds of tumor initiation, therapy resistance and metastatic spreading[138]. Thus, the identification of molecular markers based on CSCs activity could provide innovative tools to stratify mCRC patients, optimize treatment choices and identify new therapeutic targets.

PcGs are epigenetic effectors essential for stem cell self-renewal and lineage-specific gene silencing[116]. During development, they orchestrate tissue specification and body plan segmentation. PcGs are organized in multimeric Polycomb repressive complexes (PRCs), named PRC1-4. In addition to its role in stem cell biology and development, PRC2 is crucial for CSC self-renewal and tumor progression[116]. It is

made up by 4 proteins, and is able to bind specific DNA sequences to catalyze histone H3 lysine 27 trimethylation[116]. This epigenetic mark triggers gene silencing. EZH2 is the catalytic subunit of PRC2. It is essential for CSC self-renewal in several solid tumors[116,117], where it mediates E-cadherin silencing and cancer cell invasion[125]. In addition, pharmacological inhibition of EZH2 induces apoptosis in CRC cell lines, by up-regulating the apoptosis effector FBXO32[139]. Immunohistochemical analyses revealed that EZH2 is an independent prognostic indicator in CRC, and that its expression significantly increases with tumor stage[140]. In addition, high EZH2 mRNA expression predicts shorter OS in CRC patients[141]. Due to its role in CSC biology and CRC progression, EZH2 is an interesting candidate as a novel prognostic marker.

Single nucleotide polymorphisms (SNPs) have been long tested as prognostic and predictive biomarkers, since they are easy-to-detect genetic variants that can be analyzed from a peripheral blood sample. Thus, they are attractive molecular markers for translational studies. Recently, the *EZH2* locus has been mapped for the presence of SNPs in normal individuals and lung cancer patients[142]. Three *EZH2* SNPs were shown to predict lung cancer risk, while another was characterized as producing an aminoacidic change in the EZH2 protein.

Moving from the hypothesis that polymorphic variants of such a key determinant of CSCs biology may affect clinical outcome, we aimed at investigating the role of these four *EZH2* SNPs among mCRC patients treated with a modern first-line regimen such as FOLFIRI with or without bevacizumab.

3.2. Materials and methods

3.2.1. Oncomine analysis

Oncomine 4.4 database[143] collects gene expression data from cancer patients. This database was interrogated to investigate significant overlaps between two concepts: "Activated upon Polycomb Group knockdown"[144] and "Patient Treatment Response" in CRC. We found just two significant overlaps ($p<0.01$, Odds Ratio >2.0): one relative to gene expression changes after exposure to 5-FU in rectal cancer (Clarke-Colon data set), and one relative to response to FOLFIRI regimen in mCRC patients (Graudens-Colon data set). In the latter case, gene expression analysis was performed on primary tumors and metastasis of chemotherapy-naïve patients. All patients were treated with the FOLFIRI regimen, and responders and non-responders were classified according to the World Health Organization (WHO) criteria.

We aimed at investigating mCRC patients treated with a currently employed chemotherapy regimen: we therefore focused on the latter data set.

Since *EZH2* expression was not reported in Graudens-Colon, we identified two studies on stage II-III CRC patients treated with fluoropyrimidine-based chemotherapy (Jorissen Colorectal 3, and Smith Colorectal), which reported *EZH2* expression.

3.2.2. Patient selection and study treatment

We retrospectively identified patients with histologically confirmed, metastatic colorectal adenocarcinoma receiving first-line FOLFIRI with or without bevacizumab. Different schedules of the FOLFIRI regimen were allowed, either:

- irinotecan 180 mg/sqm intravenously on day 1;
- LV 200 mg/sqm intravenously on day 1;
- 5-FU 400 mg/sqm bolus intravenously on day 1, followed by 5-FU 2400 mg/sqm 46-hours continuous infusion (ci) intravenously on days 1 to 3;

or:

- irinotecan 180 mg/sqm intravenously on day 1;
- LV 200 mg/sqm intravenously on day 1;
- 5-FU 3200 mg/sqm 48-hours ci intravenously on days 1 to 3 (without bolus administration).

Bevacizumab was administered at the dose of 5 mg/kg intravenously over 30 minutes on day 1. Each cycle was repeated every 2 weeks.

Patients were considered eligible for inclusion into the study if they had received an actual dose intensity of 5-FU and irinotecan of at least 85% of the projected dose intensity.

Pretreatment evaluation included medical history, physical examination and assessment of performance status. Complete blood cell count with differential, routine chemistry, liver and kidney function tests, CEA analysis and a computed

tomography scan of the chest and abdomen were performed before treatment start and every 2 months until evidence of disease progression.

3.2.3. Evaluation of activity and efficacy

Objective response assessment was performed according to Response Evaluation Criteria In Solid Tumors version 1.1 (RECIST)[145]. The assessment of response and progression was based on investigator-reported measurements. All patients with measurable lesions were evaluated for response, while all genotyped patients were included in the PFS and OS analyses. PFS was defined as the time from the date of treatment start until the evidence of disease progression or death from any cause, whichever occurred first. Patients who underwent secondary radical surgery on metastases were censored at the time of surgery. OS was defined as the time from the date of treatment start until death from any cause.

3.2.4. Sample collection, DNA and RNA isolation

Peripheral venous blood samples from an antecubital vein of 110 mCRC patients treated with first-line FOLFIRI plus bevacizumab were collected before treatment start and stored in anonymity at -20°C in the laboratory of Pharmacology (Department of Internal Medicine, University of Pisa, Italy) until molecular analyses were performed. DNA analyses were performed by investigators who were blinded

to clinical data. Patients provided written informed consent before entering the study. The study was conducted in accordance to the Declaration of Helsinki and to the Good Clinical Practice guidelines and protocol was approved by the Ethics Committee of Pisa University Hospital. Patients were informed of the investigational nature of the study and provided their written informed consent before registration onto the study.

Genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen). The purity and quantity of DNA obtained, was measured by Uvikon-940 spectrophotometer (Kontron). A volume of 1 μl of DNA was diluted in 499 μl of autoclaved DNase-RNase-free water. DNA Absorbance was read at 260 nm, whereas protein contamination was assessed by the 260/280 nm absorbance ratio.

Both DNA and RNA were extracted from peripheral blood lymphocytes of 50 consecutive radically resected stage II or III CRC patients, as previously described[146]. Blood samples were collected before the start of adjuvant treatment. RNA was retro-transcribed, as described in previous report[146].

3.2.5. SNP genotyping and EZH2 mRNA expression

EZH2 SNPs [c.553G>C (rs2302427); c.2110+6A>C (rs41277434); c.626-394C>T (rs3757441); g.91772121T>C (rs6958683)] were analyzed through Real-Time PCR. SNPs of our interest were studied with TaqMan probe–based assays using the PCR Real-Time ABI PRISM 7900HT instrument equipped with the Sequence Detection System version 2.0 software (Applied Biosystems). Assays IDs

were: C__15757626_10 (rs2302427) and C___326857_10 (rs3757441). The remaining SNP assays have been designed through File-Builder software (Applied Biosystems). The PCR reactions were done using 20 ng of genomic DNA diluted in 11.875 µl DNase-RNase–free water, 12.5 µl of TaqMan Universal PCR Master Mix with AmpliTaq Gold, and 0.625 µl of the assay mix (forward and reverse specific primers and the specific probes), in a total volume of 25 µl. Applied Biosystems SNP genotyping assays were used for genotyping. The allelic content of each sample in the plate was determined by reading the generated fluorescence.

After retro-transcription, cDNA from a parallel cohort of 50 CRC patients was used to measure *EZH2* expression, using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as reference gene. Applied Biosystems gene expression assay numbers were: 4326317 (GAPDH) and Hs01016789_m1* (EZH2). Figure 2 shows -ΔCt values, normalized to the lowest *EZH2* expression level.

3.2.6. Statistical analysis

This retrospective analysis aimed to evaluate the relation of *EZH2* polymorphisms with outcome parameters such as:

- response rate (RR);
- PFS;
- OS.

All polymorphisms were examined for deviation from Hardy-Weinberg equilibrium[147] by comparing actual allelic distributions with those expected using a chi-square test.

A two-tailed Fisher's exact test was used to evaluate the association of investigated *EZH2* SNPs with objective response. PFS and OS were determined using the Kaplan-Meier method and compared according to *EZH2* SNPs variants using the log-rank test. Statistical significance was set at $p<0.05$ for a two-tailed test. Statistical analyses were done using GraphPad Prism (version 5) software.

We used a Cox model to evaluate the effect of 626-394C>T and prognostic factors on PFS and OS, using the survival library of the R package[b]. For PFS, the following factors were considered:

- age;
- previous adjuvant chemotherapy;
- CEA value >100 ng/mL;
- mucinous histology;
- timing of metastases;
- Köhne score.

For OS, the following factors were included in the analysis:

- gender;
- age;
- timing of metastases;
- Köhne score;

[b] Terry Therneau and original R port by Thomas Lumley (2009). Survival: survival analysis, including penalized likelihood. R package version 2.35-7. http://CRAN.R-project.org/package=survival

- lactate dehydrogenase (LDH) above the upper limit of normal (ULN);

- previous adjuvant chemotherapy;

- mucinous histology;

- secondary surgery of metastases;

- primary tumor location.

3.2.7. *In silico* characterization of the 626-394C>T SNP

In order to evaluate transcription factor (TF) binding affinity of the C and T allele, we used PROMO 3.0 software[c]. We considered only human factors and human binding sites, with a maximum matrix dissimilarity rate of 15. DNA sequence was downloaded from "Entrez SNP"[d]. Using the same DNA sequence (and its complementary inverted counterpart) we tested the hypothesis that this SNP may affect the creation of a new splice variant in exon 6 and 7. For this purpose, we employed the NCBI Aceview Database[e].

[c] http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3
[d] http://www.ncbi.nlm.nih.gov/snp
[e] http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&l=EZH2

3.3. Results

3.3.1. Polycomb targets are specifically silenced in FOLFIRI non-responders

To investigate the relationship between EZH2 activity and response to chemotherapy, we queried Oncomine 4.4 database, which collects microarray data from cancer patients[143]. In particular, we investigated if PcG targets were differentially expressed in chemotherapy-sensitive *vs.* -resistant CRC patients[148]. PcG targets are silenced by EZH2 through histone H3K27 methylation[144]. Interestingly, we found that PcG targets are silenced in FOLFIRI non-responders, compared to FOLFIRI responders ($p<0.01$, Odds 4.6; **Figure 3.1**). Keeping with this evidence, high *EZH2* expression predicts shorter recurrence-free survival and 1-year survival rate in CRC patients treated with fluoropyrimidine-based chemotherapy ($p=0.041$ on 100 patients, and $p=0.036$ on 174 patients respectively).

Thus, EZH2 seems to be more active in FOLFIRI-resistant patients. For this reason, we selected mCRC patients treated with the FOLFIRI regimen in combination with bevacizumab.

3.3.2. Patient characteristics and treatment outcome

One-hundred and ten patients treated with FOLFIRI plus bevacizumab were identified. Patient clinical characteristics are listed in **Table 3.1**. As regards activity

in the entire population, 106 patients were evaluable for response (4 patients were not evaluable because they had no measurable disease, i.e. peritoneal carcinomatosis): complete response was reported in 13 patients (12%) and partial response in 55 patients (52%), thus resulting in overall RR of 64%. Twenty-nine (27%) patients achieved disease stabilization as best response, while 9 (8%) progressed during treatment. Eight (7%) patients underwent secondary radical (R0) surgical resection of metastases.

At a median follow up of 18.9 months, 72 patients experienced disease progression (patients not evaluable for RR were included in the PFS analysis since they experienced unequivocal disease progression by the appearance of new lesions in different organs) and 40 patients have died: median PFS and OS were 9.9 and 23.3 months, respectively.

3.3.3. Genotype information

Genotype frequencies found in our population are reported in **Table 3.2**.

All polymorphisms follow Hardy-Weinberg equilibrium. Frequencies for all four polymorphisms are comparable with those reported in a previous study on a Caucasian population[142].

3.3.4. Correlation between *EZH2* SNPs and outcome

Correlations between EZH2 SNPs, RR and clinical outcome parameters are summarized in **Table 3.2**. No significant association was detected between genotypes and RR ($p>0.05$ for all comparisons). Similarly, 553G>C, 2110+6A>C and 9177211T>C variants did not show significant association with clinical outcome. As regards 626-394C>T, median PFS achieved by patients carrying the three different variants (T/T, T/C and C/C) were 11.2, 10.1, and 8.7 months, respectively ($p=0.029$). These three genotypes did not differ significantly in terms of median OS (18.3, 27.9 and 23.8 months, respectively; $p=0.148$).

We noticed a similar PFS between T/T and T/C patients (HR=1.158, 95% CI 0.690-1.945, $p=0.578$), while a statistically significant difference was reported comparing T/T or T/C with C/C patients (HR=0.260, 95% CI 0.097-0.695, $p=0.007$, and HR=0.345, 95% CI 0.134-0.890, $p=0.028$, respectively). This observation prompted us to compare clinical outcome between C/C homozygotes and subjects carrying at least one T allele. When compared with patients with at least one T allele, C/C homozygotes showed significantly shorter median PFS (11.0 *vs.* 8.7 months; HR=0.269, 95% CI: 0.102-0.706, $p=0.008$) (**Figure 3.2**) and OS (23.8 *vs.* 18.3 months; HR=0.329, 95% CI: 0.109-0.997, $p=0.049$) (**Figure 3.3**).

To corroborate our hypothesis, we analyzed the 626-394C>T SNP on an additional retrospective cohort of 104 mCRC patients treated with the FOLFIRI regimen (without bevacizumab). These patients received the same schedules of chemotherapy as administered in the FOLFIRI plus bevacizumab cohort and disease status was evaluated as specified in the Materials and Methods for the bevacizumab-

treated group. For the FOLFIRI-receiving group, multivariate analysis for PFS and OS was not performed, since only the best objective response achieved and the dates of progression and death were collected. As with FOLFIRI plus bevacizumab, also with FOLFIRI alone no significant association between *EZH2* genotype and objective response was found (probably due to the limited sample dimension: in fact, RR was 0% in CC patients and 45% in CT/TT patients, p=0.502), while both PFS (**Figure 3.4**) and OS (**Figure 3.5**) were significantly shorter in C/C patients compared to other genotypes ($p<0.05$ Log-Rank Test). Unfortunately, we found just two C/C patients in this group. This small number prevents any conclusive confirmation of our findings.

3.3.5. Cox model and interaction test

In order to test the hypothesis that the 626-394C>T SNP is an independent prognostic factor in our population, we performed a Cox regression including all variables known to possibly affect PFS or OS n mCRC patients. At multivariate analysis, C/C genotype retained its significant association with worse PFS (HR=2.211, 95% CI: 1.057-4.624; p=0.035) (**Table 3.3**). This difference reflected into a significantly shorter OS (HR=2.851, 95% CI: 1.024-7.938; p=0.045) (**Table 3.3**). Another factor found to be associated with worse PFS and OS in our series was mucinous histology.

Due to the small number of C/C patients found in each group, we also conducted a multivariate analysis including all 214 patients (treated with FOLFIRI

with or without bevacizumab). Analysis variables included therapy regimen (FOLFIRI with or without bevacizumab) and *EZH2* genotype. This analysis included a total of 14 C/C patients. As shown in **Table 3.4**, C/C genotype was an independent predictor of both PFS and OS. As expected, FOLFIRI plus bevacizumab regimen was associated with a significant survival advantage. We also tested if *EZH2* genotype predicted a benefit from adding bevacizumab to the FOLFIRI regimen (genotype-treatment interaction). As shown in **Table 3.4**, *EZH2* genotype was not predictive of bevacizumab efficacy ($p>0.05$ for both PFS and OS).

3.3.6. *In silico* and *in vivo* characterization of the 626-394C>T polymorphism

The 626-394C>T SNP was reported as an intronic polymorphism, located between exons 6 and 7[142]. Polymorphic variants located in uncoding regions may affect gene functions in many ways, including affinity changes in TF binding sites, and alteration of specific splice regions. Due to the putative role of this variant in mCRC patients, we investigated if the T-C change at this residue may alter TF binding or splicing sites in exons 6 and 7. For this purpose, we used PROMO 3.0 software and NCBI Aceview database. The latter tool revealed that 23 *EZH2* splice variants have been described. No one of these includes the residue hosting the 626-394C>T variant.

We then evaluated TF binding affected by the C-T variant. As shown in **Figure 3.6**, the T allele may create a binding site for the XBP1 factor.

To confirm our prediction, we measured *EZH2* expression in peripheral lymphocytes from 50 consecutive CRC patients. We also analyzed the 626-394C>T SNP in these patients. As shown in **Figure 3.7**, the C/C variant was associated with a significantly higher EZH2 expression, with respect to C/T and T/T genotypes ($p<0.05$). Interestingly, EZH2 levels were not significantly different between C/T and T/T genotypes.

Table 3.1. Patient characteristics

Characteristic	***N***	***%***
No. of patients	110	100%
Age, years		
Median (range)	63 (35-79)	
Gender		
Male	57	52%
Female	53	48%
ECOG Performance Status		
0	101	92%
1	8	7%
2	1	1%
Primary tumor		
Colon	82	75%
Rectum	28	25%
Timing of metastases		
Synchronous	61	55%
Metachronous	49	45%
No. of involved organs		
1	56	51%
2	46	42%
≥3	8	7%
Previous adjuvant chemotherapy		
Yes	44	40%
No	66	60%

*Köhne prognostic group**		
Low risk	56	52%
Intermediate risk	39	36%
High risk	12	11%

Abbreviations: No., number; ECOG, Eastern Cooperative Oncology Group. *For 3 patients Köhne score was not assessable due to missing data.

3.2. EZH2 polymorphisms: association with RR, PFS and OS

Genotype	No. (%)	RR (%)	*p*	Median PFS (months)	*p*	Median OS (months)	*p*
553G>C							
C/C	96 (87%)	59 (61%)		10.2		23.3	
G/C	13 (12%)	9 (69%)	*0.587*	10.4	*0.820*	25.4	*0.893*
G/G	1 (1%)	NE		undefined		undefined	
2110+6T>G							
A/A	95 (86%)	60 (63%)		10.2		23.1	
A/C	13 (12%)	7 (54%)	*0.469*	10.5	*0.695*	29.6	*0.739*
C/C	2 (2%)	1 (50%)		undefined		undefined	
9177211T>C							
C/C	74 (67%)	43 (58%)		8.7		23.8	
T/C	33 (30%)	23 (70%)	*0.287*	9.5	*0.189*	21.6	*0.314*
T/T	3 (3%)	2 (67%)		11.1		18.3	
626-394C>T							
T/T	62 (56%)	39 (63%)		11.2		23.8	
T/C	36 (33%)	22 (61%)	*0.754*	10.1	*0.029*	27.9	*0.148*
C/C	12 (11%)	7 (58%)		8.7		18.3	

Abbreviations: NE, not evaluable.

3.3. Multivariate analysis: FOLFIRI plus bevacizumab cohort

Progression-free survival			
Factors	**HR**	**95% CI**	***p***
Age	0.993	0.969-1.018	*0.596*
Adjuvant chemotherapy			
Yes			
No	0.286	0.077-1.055	*0.060*
CEA value >100 ng/mL			
No			
Yes	0.760	0.385-1.498	*0.427*
Mucinous histology			
No			
Yes	2.492	1.173-5.293	*0.018**
Timing of metastases			
Metachronous			
Synchronous	2.096	0.551-7.980	*0.278*
Köhne score			
High			
Intermediate	0.728	0.314-1.689	*0.460*
Low	0.752	0.341-1.660	*0.481*
626-394C>T			
T/T or T/C			
C/C	2.211	1.057-4.624	*0.035**

Overall survival			
Factors	**HR**	**95% CI**	***p***
Gender			
Female			
Male	1.147	0.476-2.763	*0.761*
Age	1.007	0.962-1.054	*0.755*
Timing of metastases			
Metachronous			
Synchronous	1.641	0.452-5.958	*0.451*
Köhne score			
High			
Intermediate	0.574	0.178-1.851	*0.353*
Low	0.610	0.188-1.978	*0.410*
LDH level >ULN			
No			
Yes	2.138	0.839-5.450	*0.111*
Mucinous histology			
No			
Yes	5.511	2.119-14.334	*<0.001**
Secondary surgery of metastases			
No			
Yes	0.151	0.014-1.655	*0.122*
Adjuvant chemotherapy			
Yes			
No	0.444	0.122-1.623	*0.220*
Primary tumor site			

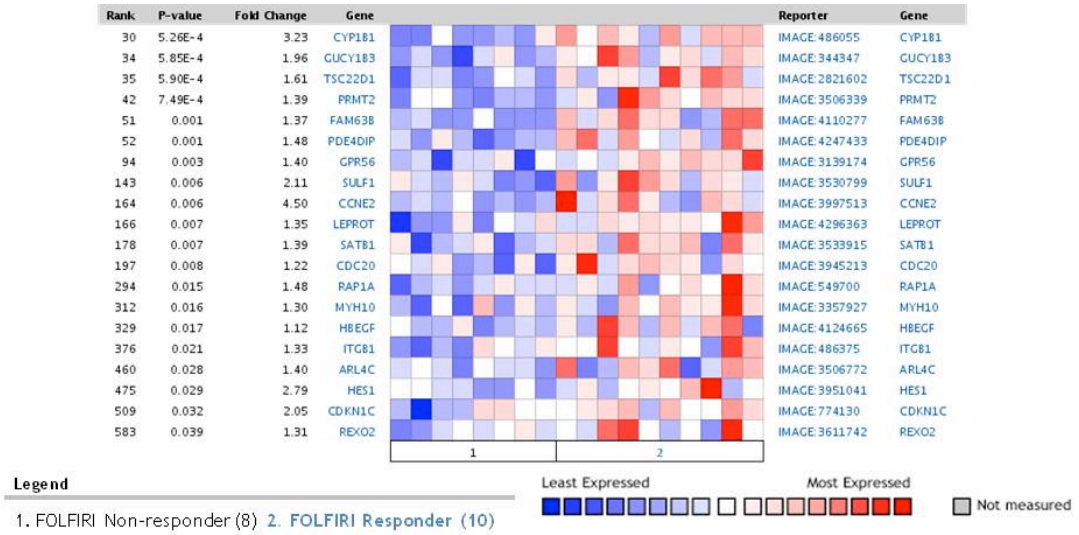
Colon			
Rectum	0.852	0.329-2.207	*0.742*
626-394C>T			
T/T or T/C			
C/C	2.851	1.024-7.938	*0.045**

Abbreviations: ECOG, Eastern Cooperative Oncology Group; ULN, upper limit of normal.

Table 3.4. Multivariate analysis: FOLFIRI with or without bevacizumab

Factors	HR	95% CI	*p*
Progression-free survival			
626-394C>T			
T/C or T/T			
C/C	5.752	1.358-24.369	*0.018**
Treatment			
FOLFIRI			
FOLFIRI plus bevacizumab	0.401	0.268-0.601	*<0.001**
Genotype-treatment interaction	0.476	0.092-2.478	*0.378*
Overall survival			
626-394C>T			
C/C			
T/C or T/T	6.091	1.447-25.635	*0.014**
Treatment			
FOLFIRI			
FOLFIRI plus bevacizumab	0.602	0.398-0.910	*0.016**
Genotype-treatment interaction	0.334	0.064 -1.738	*0.193*

Figure 3.1. PcG targets down-regulated in FOLFIRI non-responders



For analysis settings, see "Material and Methods". Using our filters, we found 2 significant overlaps: one relative to gene expression changes after exposure to 5-FU in rectal cancer (Clarke-Colon data set), and one relative to response to FOLFIRI regimen in mCRC patients (Graudens-Colon data set). In the latter case, gene expression analysis was performed on primary tumors and metastasis of chemotherapy-naïve patients. All patients were treated with FOLFIRI regimen. Responders and non-responders were classified according to WHO criteria. Since we aimed at investigating mCRC patients treated with a currently employed chemotherapy regimen, we focused on the latter data set.

The figure shows that most PcG targets are silenced in FOLFIRI non-responders ($p<0.01$, Oncomine analysis).

Figure 3.2. PFS and *EZH2* genotype in the FOLFIRI plus bevacizumab cohort

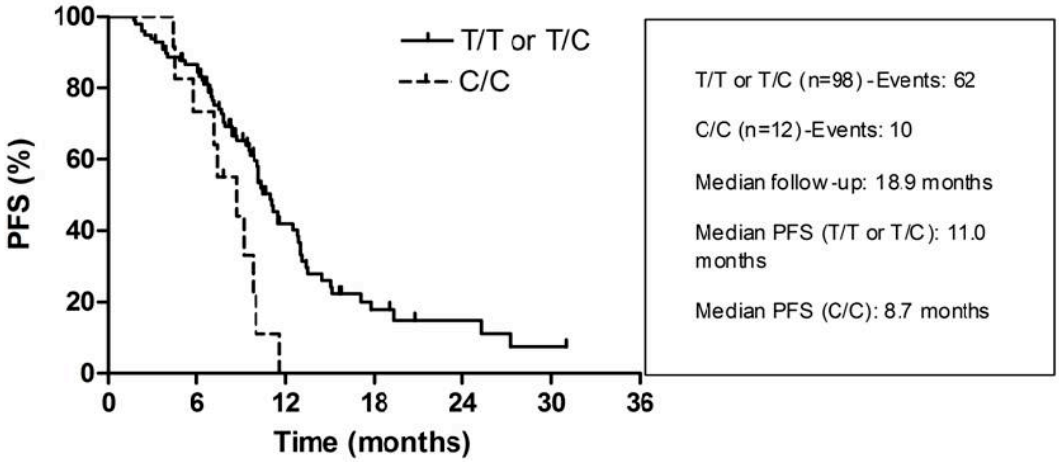


Figure 3.3. OS and *EZH2* genotype in the FOLFIRI plus bevacizumab cohort

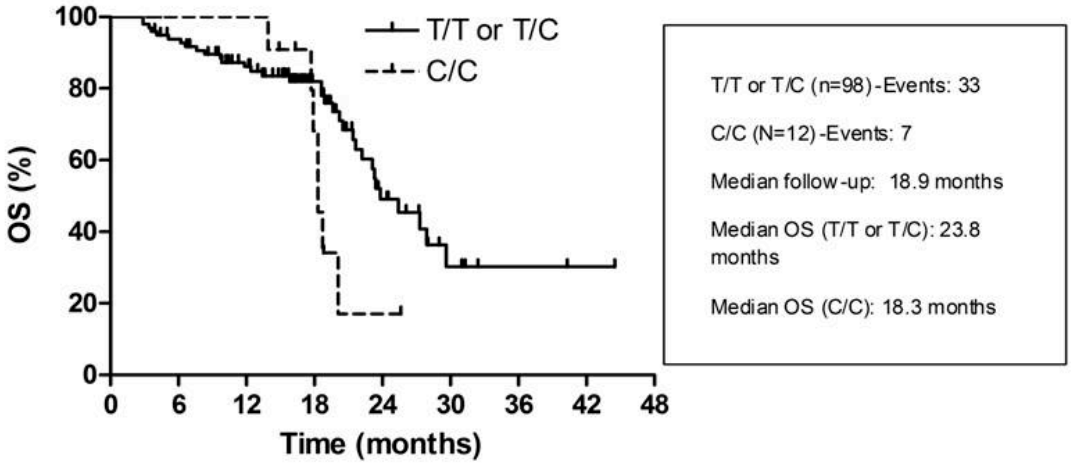


Figure 3.4. PFS and *EZH2* genotype in the FOLFIRI cohort

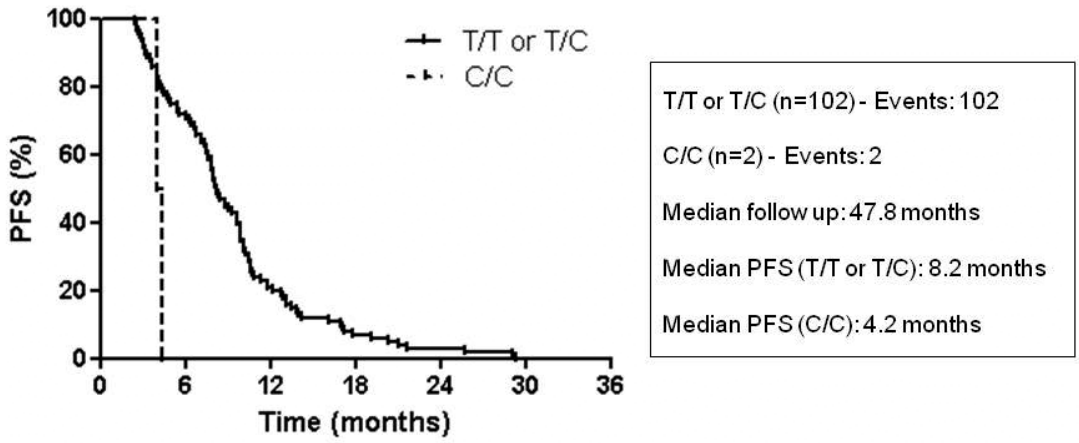


Figure 3.5. OS and *EZH2* genotype in the FOLFIRI cohort

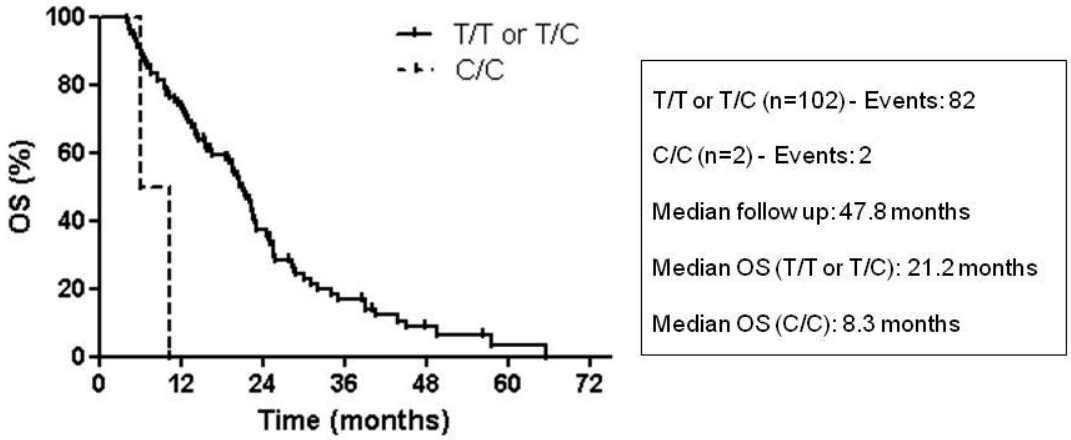
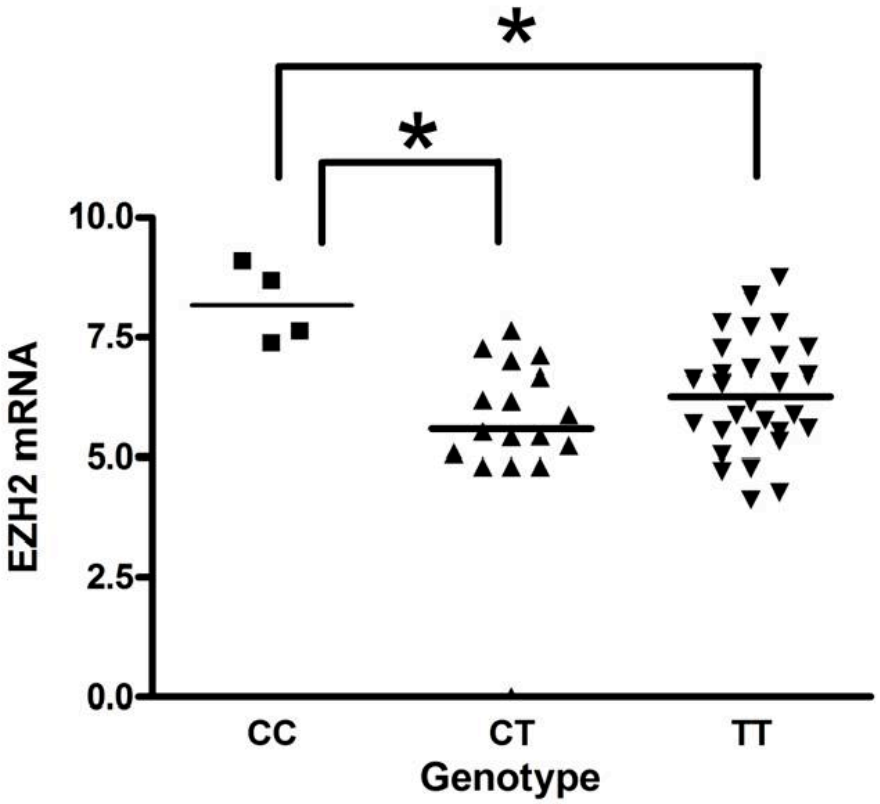


Figure 3.6. Characterization of TF binding sites affected by the rs3757441 SNP

Characterization of TF binding sites affected by the rs3757441 SNP. Prediction on XBP binding are performed through PROMO3.0 software, as described in "Materials and Methods". In red, XBP consensus sequence.

Figure 3.7. EZH2 mRNA levels in resected CRC patients



Gene expression in lymphocytes is relative to GAPDH levels. *p<0.05 (ANOVA, Bonferroni post-test).

CHAPTER 4. *DISCUSSION AND CONCLUSIONS*

4.1. Discussion

Our analysis shows that the 626-394C>T *EZH2* polymorphism is associated with shorter PFS and OS in a group of mCRC patients treated with the FOLFIRI (with or without bevacizumab) regimen. *EZH2* is emerging as a novel oncogene and putative therapy target in oncology[149]. Along with its role in CSC self-renewal (17), *EZH2* is known to silence several tumor-suppressor genes, including *CDH1* (E-cadherin) and *p16INK4A*/*p14ARF*[125,150]. E-cadherin is an epithelial surface molecule, that regulates cell adhesion and cell growth in CRC cells[151]. In the gastrointestinal tract, E-cadherin silencing is the first step for epithelial-to-mesenchymal transition and cancer cell invasion[152]. In addition, E-cadherin down-regulation contributes to Wnt pathway activation[153], that in turn triggers 5-FU and irinotecan resistance genes in CRC cells[154]. The *p16INK4A*/*p14ARF* locus encodes for two cell-cycle inhibitors that activate senescence in response to cellular stress[150]. In CRC, this locus is a frequent target of epigenetic silencing[155]. This phenomenon leads to uncontrolled proliferation and resistance to chemotherapy[156]. Thus, *EZH2* may contribute to CRC progression and treatment resistance. Keeping with this hypothesis, high *EZH2* expression has been associated with shorter OS and higher stage in CRC patients[140,141]. In particular, Wang et al.[140] showed that EZH2 protein expression is an independent predictor of poor prognosis in CRC.

The 626-394C>T SNP has been previously characterized as an intronic polymorphism[142]. Intronic SNPs may affect gene expression through several mechanisms, including changes in TF binding sites[157], microRNA target sequences[158], and splicing variants[159]. This SNP is not located on the 3' UTR region, thus it does not likely affect microRNA binding. In addition, we found that the residue hosting the 626-394C>T variant is not involved in alternative splicing. Thus, a mechanism by which this SNP may affect *EZH2* expression is through TF binding. Our *in silico* analysis revealed that the T allele may create a binding site for the XBP1 factor (**Figure 3.6**). Patients carrying the C/C genotype have no binding sites for this factor. XBP1 is a basic leucin zipper (b-zip) TF[160], which is activated by cellular stress (i.e. hypoxia, DNA damage). XBP1 dimerizes with other b-zip proteins, thereby binding specific DNA sequences. XBP1-containing heterodimers may lead to activation and inhibition of different set of genes, depending on the cellular context[161]. For example, in pancreatic cells, XBP1 inhibits Pdx and Mafa genes, thereby suppressing insulin production[162]. XBP1 is expressed by CRC cells, and its up-regulation leads to cell death[163]. Interestingly, DNA damage is known to trigger *EZH2* down-regulation[164]. It is conceivable that, in response to cellular stress, XPB1 inhibits *EZH2* expression in CRC cells. In patients carrying at least one T allele, *EZH2* expression may be inhibited by XBP1. In C/C homozigotes, *EHZ2* expression could be deregulated, thereby producing more aggressive tumors. Keeping with this hypothesis, we found that *EZH2* mRNA levels are higher in C/C patients, compared to other genotypes (**Figure 3.7**). The C/T and T/T genotypes did not show a significantly different *EZH2* expression. Thus, our data suggest that C/C homozygous individuals show both poorer prognosis, and higher *EZH2* expression.

Interestingly, heterozygous genotype was not associated to intermediate *EZH2* expression and prognosis. Several regulatory mechanisms may account for the same expression levels in C/T and T/T individuals. For example XBP1 up-regulation may reduce *EZH2* expression in C/T, but not in C/C patients (lacking XBP1 binding site). For other genetic variants, it has been reported that homozygous wild-type and heterozygous individuals show identical gene expression levels, while only homozygous mutant individuals demonstrate significantly different expression[165].

It is worth noting that other mechanisms may explain the prognostic role of the 626-394C>T variant in mCRC patients. For example, the 626-394C>T variant is in linkage disequilibrium with several other EZH2 polymorphisms[142] that may play a more determinant functional role. Thus, the *in silico* prediction needs to be mechanistically confirmed. Moreover, the impact of this variant on EZH2 expression in CRC cells should be investigated in future studies.

Reported results show that patients carrying zero, one or two C alleles reported a progressively worse PFS, while the same trend was not significant for OS. Nonetheless, the worst OS outcome was observed in C/C homozygotes. Since PFS more accurately reflects the benefit of first-line treatment than OS, the correlation we found in our series may suggest that the polymorphic variant has a main predictive effect on treatment benefit. Even though we did not observe significant correlation between 626-394C>T polymorphism and treatment activity, this could be partly explained by the high RR (64%) in our retrospective series. However, median PFS and OS times in our population are in line with those reported in randomized and observational trials[7] with FOLFIRI plus bevacizumab, thus reducing the risks of major selection bias. It is worth noting that comparing the C/C homozygotes with all

other genotypes we found a significant association with both PFS and OS. More importantly, the C/C genotype emerged as an independent predictor of poorer prognosis at multivariate analysis. Moreover, the C/C variant seemed to predict PFS and OS with higher sensitivity than other conventional prognostic variables.

Interestingly, mucinous histology was significantly associated with worse PFS and OS at multivariate analysis. These results seem to confirm previous reports suggesting the impact of mucinous histology on both benefit from treatment and OS in mCRC patients treated with first-line chemotherapy[166,167].

To conclude, this is the first experience suggesting that an *EZH2* polymorphism has significant impact on clinical outcome in oncology. We reported a correlation between the 626-394C>T SNP and both PFS and OS in mCRC patients treated with the FOLFIRI regimen in first-line. Since we found that only C/C genotype is associated with worse prognosis, a major limit of this study is the reduced number of C/C individuals evaluated (12 in the FOLFIRI plus bevacizumab group and only 2 in the FOLFIRI alone group). We tried to overcome this limit by including all 214 patients in a multivariate analysis (**Table 3.4**). This analysis showed that the *EZH2* genotype is an independent predictor of clinical outcome in mCRC patients treated with the FOLFIRI regimen (with or without bevacizumab). Moreover, no significant interaction between the *EZH2* genotype and treatment was reported: this evidence seems to strengthen a main prognostic value or, on the other hand, a potential predictive role in patients receiving irinotecan-based chemotherapy (as also suggested by the Oncomine analysis) of the studied *EZH2* SNP. However, these results need to be confirmed by larger, prospective independent series in order to overcome possible bias inherent to retrospective evaluations. In addition, the same

SNP should be tested on mCRC patients treated with other regimens (e.g. oxaliplatin-based combinations) to discriminate between predictive and prognostic power.

4.2. Conclusions and future development

The evidence supporting a role for CSCs in solid tumors is fascinating, since it opens new frontiers for the development of novel anticancer agents, prediction of benefit from treatment and prognostic stratification of patients: the CSC hypothesis thus sheds new light on how we interpret carcinogenesis, tumor recurrence and mechanisms of resistance to medical treatment. *EZH2* is a key element in transcriptional regulation of CSCs and therefore is an intriguing candidate for translational analysis and drug development. This is the first analysis of the role of *EZH2* SNPs in mCRC patients treated with first-line chemotherapy: far from being conclusive, this pivotal experience leaves some unanswered questions and opens new fields of investigation.

In particular, the hypothesis that allelic variants of the 626-394C>T SNP might affect gene transcription (partly supported by the *in vivo* evaluation of EZH2 mRNA levels) should be confirmed by direct measurement of EZH2 protein levels in tumor tissue: this analysis will provide conclusive evidence that this SNP variants affect *EZH2* function through differential protein expression. As previously discussed, however, other mechanisms may explain differential function of *EZH2* according to the 626-394C>T SNP genotype: for example, the *in silico* characterization suggests

that EZH2 binding site to transcription factors may be altered in patients harbouring the C variant, thus affecting the functional role of *EZH2* in CSCs. Moreover, as discussed in the previous chapter, EZH2 levels may be influenced by other mechanisms, as well as EZH2 function may be altered by somatic mutations (as described in haematologic malignancies). All these alternative mechanisms should be further investigated.

These preliminary data suggest that the 626-394C>T SNP has a main prognostic value: SNP evaluation on larger series of mCRC patients treated with other chemotherapy regimens than FOLFIRI may therefore be of interest to confirm this finding. Even more importantly, adequately powered prospective trials should be conducted to definitively validate this parameter as a major prognostic indicator in mCRC.

As discussed in the first chapter, other prognostic molecular biomarkers may be of clinical usefulness in mCRC, particularly *BRAF* V600E mutation. Some preliminary laboratory findings suggest that *EZH2* is linked to the *RAF* signalling pathway: in fact, Chang et al. found that in breast tumor initiating cells *EZH2* expression-mediated downregulation of DNA damage repair leads to accumulation of recurrent *RAF1* gene amplification, which in turn activates p-ERK-β-catenin signaling to promote breast CSC expansion[168]. Moving from these preclinical evidence and the compelling demonstration that *BRAF* mutant colorectal tumors have a particularly poor prognosis, investigating the correlation of *EZH2* expression and genotype and *BRAF* mutational status may be of extreme interest, since it may help to clarify the mechanisms underlining the aggressiveness of such cancers and to open new options for the treatment of *BRAF* mutant cases.

Finally, recent studies have underlined a potential role of putative CSC markers in determining prognosis of patients after radical resection of non-metastatic disease. However, these analyses suffer from several limitations, due to inadequate sample dimension, technical difficulties and only rough identification of CSC. Therefore, adding some *bona fide* markers of CSC behaviour to "conventional" CSC markers might implement the prognostic stratification of radically resected patients. Moreover, since CSCs are considered the seeds of metastatic spreading, CSC-based biomarkers might identify resistant tumors which are insensitive to adjuvant chemotherapy, thus helping clinicians in refining treatment choices.

References

1. Jemal A, Bray F, Center MM et al. Global cancer statistics. *CA Cancer J Clin* 2011; 61: 69-90. Erratum in: *CA Cancer J Clin* 2011; 61: 134.

2. La Vecchia C, Bosetti C, Lucchini F et al. Cancer mortality in Europe, 2000-2004, and an overview of trends since 1975. *Ann Oncol* 2010; 21: 1323-1360.

3. Horner MJ, Ries LAG, Krapcho M et al. SEER cancer statistics review, 1975–2006. Bethesda, MD: National Cancer Institute; http://seercancergov/csr/1975_2006/; 2009.

4. Kopetz S, Chang GJ, Overman MJ et al. Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. *J Clin Oncol* 2009; 27: 3677-3683.

5. Adam R, Wicherts DA, de Haas RJ et al. Patients with initially unresectable colorectal liver metastases: is there a possibility of cure? *J Clin Oncol* 2009; 27: 1829-1835.

6. Loupakis F, Masi G, Vasile E, Falcone A. First-line chemotherapy in metastatic colorectal cancer: new approaches and therapeutic algorithms. Always hit hard first? *Curr Opin Oncol* 2008; 20: 459-465.

7. Fornaro L, Masi G, Loupakis F et al. Palliative treatment of unresectable metastatic colorectal cancer. *Expert Opin Pharmacother* 2010; 11: 63-77.

8. Jonker DJ, Maroun JA, Kocha W et al. Survival benefit of chemotherapy in metastatic colorectal cancer: a meta-analysis of randomised controlled trials. *Br J Cancer* 2000; 82: 1789-1794.

9. Nordic Gastrointestinal Tumor Adjuvant Therapy Group. Expectancy or primary chemotherapy in patients with advanced asymptomatic colorectal cancer: a randomized trial. *J Clin Oncol* 1992; 10: 904-911.

10. Douillard JY, Cunningham D, Roth AD et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicenter randomized trial. *Lancet* 2000; 355: 1041-1047.

11. Saltz LB, Cox JV, Blanke C et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2000; 343: 905-914.

12. Giacchetti S, Perpoint B, Zidani R et al. Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil–leucovorin as first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 2000; 181: 136-147.

13. De Gramont A, Figer A, Seymour M et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 2000; 18: 2938-2947.

14. Giacchetti S, Itzhaki M, Gruia G et al. Long-term survival of patients with unresectable colorectal cancer liver metastases following infusional chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and surgery. *Ann Oncol* 1999; 10: 663-669.

15. Adam R, Avisar E, Ariche A et al. Five-year survival following hepatic resection after neoadjuvant therapy for nonresectable colorectal. *Ann Surg Oncol* 2001; 8: 347-353.

16. Buyse M, Thirion P, Carlson RW et al. Relation between tumour response to first-line chemotherapy and survival in advanced colorectal cancer: a meta-analysis. *Lancet* 2000; 356: 373-378.

17. Tournigand C, Andre T, Achille E et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* 2004; 22: 229-237.

18. Goldberg RM, Sargent DJ, Morton RF et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. *J Clin Oncol* 2004; 22: 23-30.

19. Grothey A, Sargent D, Goldberg RM et al. Survival of patients with advanced colorectal cancer improves with the availability of fluorouracil–leucovorin, irinotecan, and oxaliplatin in the course of treatment. *J Clin Oncol* 2004; 22: 1209-1214.

20. Arkenau HT, Arnold D, Cassidy J et al. Efficacy of oxaliplatin plus capecitabine or infusional fluorouracil/leucovorin in patients with metastatic colorectal cancer: a pooled analysis of randomized trials. *J Clin Oncol* 2008; 26: 5910-5917.

21. Kohne CH, De Greve J, Hartmann JT et al. Irinotecan combined with infusional 5-fluorouracil/folinic acid or capecitabine plus celecoxib or placebo in the first-line treatment of patients with metastatic colorectal cancer. EORTC study 40015. *Ann Oncol* 2008; 19: 920-926.

22. Fuchs CS, Marshall J, Mitchell E et al. Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: results from the BICC-C study. *J Clin Oncol* 2007; 25: 4779-4786.

23. Fuchs CS, Marshall J, Barrueco J. Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: updated results from the BICC-C study. *J Clin Oncol* 2008; 26: 689-690.

24. Koopman M, Antonini NF, Douma J et al. Sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in advanced colorectal cancer (CAIRO): a phase III randomised controlled trial. *Lancet* 2007; 370: 135-142.

25. Punt CJA, Koopman M. Capecitabine and irinotecan as first-line treatment of advanced colorectal cancer. *J Clin Oncol* 2008; 26: 1907-1908.

26. Folprecht G, Grothey A, Alberts S et al. Neoadjuvant treatment of unresectable colorectal liver metastases: correlation between tumour response and resection rates. *Ann Oncol* 2005; 16: 1311-1319.

27. Falcone A, Masi G, Allegrini G et al. Biweekly chemotherapy with oxaliplatin, irinotecan, infusional Fluorouracil, and leucovorin: a pilot study in patients with metastatic colorectal cancer. *J Clin Oncol* 2002; 20: 4006-4014.

28. Masi G, Allegrini G, Cupini S et al. First-line treatment of metastatic colorectal cancer with irinotecan, oxaliplatin and 5-fluorouracil/leucovorin (FOLFOXIRI): results of a phase II study with a simplified biweekly schedule. *Ann Oncol* 2004; 15: 1766-72.

29. Falcone A, Ricci S, Brunetti I et al. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. *J Clin Oncol* 2007; 25: 1670-1676.

30. Masi G, Vasile E, Loupakis F et al. Randomized trial of two induction chemotherapy regimens in metastatic colorectal cancer: an updated analysis. *J Natl Cancer Inst* 2011; 103: 21-30.

31. Souglakos J, Androulakis N, Syrigos K et al. FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) vs FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) as first-line treatment in metastatic colorectal cancer (MCC): a multicentre randomised phase III trial from the Hellenic Oncology Research Group (HORG). *Br J Cancer* 2006; 94: 798-805.

32. Golfinopoulos V, Salanti G, Pavlidis N, Ioannidis JP. Survival and disease-progression benefits with treatment regimens for advanced colorectal cancer: a meta-analysis. *Lancet Oncol* 2007; 8: 898-911.

33. Masi G, Loupakis F, Pollina L et al. Long-term outcome of initially unresectable metastatic colorectal cancer patients treated with 5-fluorouracil/leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) followed by radical surgery of metastases. *Ann Surg* 2009; 249: 420-425.

34. Seymour MT, Maughan TS, Ledermann JA et al. Different strategies of sequential and combination chemotherapy for patients with poor prognosis advanced colorectal cancer (MRC FOCUS): a randomised controlled trial. *Lancet* 2007; 370: 143-152.

35. Seymour MT, Thompson LC, Wasan HS et al. Chemotherapy options in elderly and frail patients with metastatic colorectal cancer (MRC FOCUS2): an open-label, randomised factorial trial. *Lancet* 2011; 377: 1749-1759.

36. Tournigand C, Cervantes A, Figer A et al. OPTIMOX1: a randomized study of FOLFOX4 or FOLFOX7 with oxaliplatin in a stop-and-Go fashion in advanced colorectal cancer - a GERCOR study. *J Clin Oncol* 2006; 24: 394-400.

37. Chibaudel B, Maindrault-Goebel F, Lledo G et al. Can chemotherapy be discontinued in unresectable metastatic colorectal cancer? The GERCOR OPTIMOX2 study. *J Clin Oncol* 2009; 27: 5727-5733.

38. Adams RA, Meade AM, Seymour MT et al.; on behalf of the MRC COIN Trial Investigators. Intermittent versus continuous oxaliplatin and fluoropyrimidine combination chemotherapy for first-line treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet Oncol* 2011; 12: 642-653.

39. Labianca R, Sobrero A, Isa L et al. Intermittent versus continuous chemotherapy in advanced colorectal cancer: a randomised 'GISCAD' trial. *Ann Oncol* 2011; 22: 1236-1242.

40. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; 9: 669-676.

41. Kerbel RS. Tumor angiogenesis. *N Engl J Med* 2008; 358: 2039-2049.

42. Presta LG, Chen H, O'Connor SJ et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997; 57: 4593-4599.

43. Hurwitz H, Fehrenbacher L, Novotny W et al. Bevacizumab plus irinotecan, fluorouracil and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; 350: 2335-2342.

44. Saltz LB, Clarke S, Díaz-Rubio E et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. *J Clin Oncol* 2008; 26: 2013-2019.

45. Giantonio BJ, Meropol NJ, Catalano PJ et al. Magnitude of progression-free survival (PFS) improvement and treatment (Tx) duration in metastatic colorectal

cancer (mCRC) for bevacizumab (BV) in combination with oxaliplatin-containing regimens: An analysis of two phase III studies. *J Clin Oncol* 25, 2007 (suppl; abstr 4073).

46. Tabernero J, Aranda E, Gomez A et al. Phase III study of first-line XELOX plus bevacizumab (BEV) for 6 cycles followed by XELOX plus BEV or single-agent (s/a) BEV as maintenance therapy in patients (pts) with metastatic colorectal cancer (mCRC): The MACRO Trial (Spanish Cooperative Group for the Treatment of Digestive Tumors [TTD]). *J Clin Oncol* 28: 15s, 2010 (suppl; abstr 3501).

47. Masi G, Loupakis F, Salvatore L et al. Bevacizumab with FOLFOXIRI (irinotecan, oxaliplatin, fluorouracil, and folinate) as first-line treatment for metastatic colorectal cancer: a phase 2 trial. *Lancet Oncol* 2010; 11: 845-852.

48. Harris TJ, McCormick F. The molecular pathology of cancer. *Nat Rev Clin Oncol* 2010; 7: 251-265.

49. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. *N Engl J Med* 2008; 358: 1160-1174.

50. Normanno N, Tejpar S, Morgillo F et al. Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat Rev Clin Oncol* 2009; 6: 519-527.

51. Van Cutsem E, Köhne CH, Hitre E et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009; 360: 1408-1417.

52. Van Cutsem E, Köhne CH, Láng I et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011; 29: 2011-2019.

53. Van Cutsem E, Bokemeyer C, Heeger S et al. Outcome according to metastatic site in patients with KRAS wild-type tumors: Analysis from the CRYSTAL and OPUS studies. *J Clin Oncol* 29: 2011 (suppl 4; abstr 472).

54. Bokemeyer C, Bondarenko I, Makhson A et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 2009; 27: 663-671.

55. Bokemeyer C, Bondarenko I, Hartmann JT et al. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. *Ann Oncol* 2011; 22: 1535-1546.

56. Folprecht G, Gruenberger T, Bechstein WO et al. Tumour response and secondary resectability of colorectal liver metastases following neoadjuvant chemotherapy with cetuximab: the CELIM randomised phase 2 trial. *Lancet Oncol* 2010; 11: 38-47.

57. Maughan TS, Adams RA, Smith CG, et al.; on behalf of the MRC COIN Trial Investigators. Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet* 2011; 377: 2103-2114.

58. Tveit K, Guren T, Glimelius B et al. Randomized phase III study of 5-fluorouracil/folinate/oxaliplatin given continuously or intermittently with or without cetuximab, as first-line treatment of metastatic colorectal cancer: The NORDIC VII study (NCT00145314), by the Nordic Colorectal Cancer. *Ann Oncol* 2010, 21 (suppl 8); LBA20.

59. Garufi C, Torsello A, Tumolo S et al. Cetuximab plus chronomodulated irinotecan, 5-fluorouracil, leucovorin and oxaliplatin as neoadjuvant chemotherapy in colorectal liver metastases: POCHER trial. *Br J Cancer* 2010; 103: 1542-1547.

60. Ychou M, Desseigne F, Thezenas S et al. Preliminary results of a multicentre phase II trial evaluating cetuximab in combination with FOLFIRINOX (LV5FU + Irinotecan + Oxaliplatin) as first line treatment of metastatic colorectal cancer (mCRC) patients. *2009 Gastrointestinal Cancers Symposium*, abs 450.

61. Douillard JY, Siena S, Cassidy J et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 2010; 28: 4697-4705.

62. Walther A, Johnstone E, Swanton C et al. Genetic prognostic and predictive markers in colorectal cancer. *Nat Rev Cancer* 2009; 9: 489-499.

63. Winder T, Lenz HJ. Molecular predictive and prognostic markers in colon cancer. *Cancer Treat Rev* 2010; 36: 550-556.

64. Cunningham D, Humblet Y, Siena S et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004; 351: 337-345.

65. Chung KY, Shia J, Kemeny NE et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 2005; 23: 1803-1810.

66. Siena S, Sartore-Bianchi A, Di Nicolantonio F et al. Biomarkers predicting clinical outcome of epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer. *J Natl Cancer Inst* 2009; 101: 1308-1324.

67. Rodriguez-Viciana P, Warne PH, Dhand R et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* 1994; 370: 527-532.

68. Allegra CJ, Jessup JM, Somerfield MR et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009; 27: 2091-2096.

69. Andreyev H.J., Norman A.R., Cunningham D et al. Kirsten Ras mutations in patients with colorectal cancer: The 'Rascal II' study. *Br J Cancer* 2001; 85: 692-696.

70. Lievre A., Bachet J.B., Le Corre D et al. Kras mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 2006; 66: 3992-3995.

71. De Roock W., Piessevaux H., De Schutter J et al. Kras wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann Oncol* 2007; 19: 508-515.

72. Di Fiore F, Blanchard F, Charbonnier F et al. Clinical relevance of Kras mutation detection in metastatic colorectal cancer treated by cetuximab plus chemotherapy. *Br J Cancer* 2007; 96: 1166-1169.

73. Khambata-Ford S, Garrett CR, Meropol NJ et al. Expression of epiregulin and amphiregulin and K-Ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007; 25: 3230-3237.

74. Lievre A., Bachet J.B., Boige V et al. Kras mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 2008; 26: 374-379.

75. Karapetis CS, Khambata-Ford S, Jonker DJ et al. K-Ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008 359: 1757-1765.

76. Amado R.G., Wolf M., Peeters M. et al. Wild-type Kras is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; 26: 1626-1634.

77. EMEA: EPARs for authorised medicinal products for human use, Vectibix. http://www.emea.europa.eu/pdfs/human/opinion/40511307en.pdf.

78. EMEA: Committee for Medicinal Products for Human Use post-authorisation summary of positive opinion for Erbitux. http://www.emea.europa.eu/pdfs/human/opinion/Erbitux_28040208en.pdf.

79. Linardou H, Dahabreh I, Kanaloupiti D et al. Assessment of somatic K-Ras mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol* 2008; 9: 962-972.

80. Edkins S, O'meara S, Parker A et al. Recurrent Kras codon 146 mutations in human colorectal cancer. *Cancer Biol Ther* 2006; 5: 928-932.

81. Loupakis F, Ruzzo A, Cremolini C et al. KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. *Br J Cancer* 2009; 101: 715-721.

82. De Roock W, Claes B, Bernasconi D et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 2010; 11: 753-762.

83. De Roock W, Jonker DJ, Di Nicolantonio F et al. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 2010; 304: 1812-1820.

84. Tejpar S, Bokemeyer C, Celik I et al. Influence of KRAS G13D mutations on outcome in patients with metastatic colorectal cancer (mCRC) treated with first-line chemotherapy with or without cetuximab. *J Clin Oncol* 29: 2011 (suppl; abstr 3511).

85. Roock WD, Vriendt VD, Normanno N et al. KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. *Lancet Oncol* 2011; 12: 594-603.

86. Roth AD, Tejpar S, Delorenzi M et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. *J Clin Oncol* 2010; 28: 466-474.

87. Laurent-Puig P, Cayre A, Manceau G et al. Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. *J Clin Oncol* 2009; 27: 5924-5930.

88. Di Nicolantonio F, Martini M, Molinari F et al. BRAF wild-type is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008; 26: 5705-5712.

89. Peeters M, Oliner KS, Parker A et al. Use of massively parallel, next-generation sequencing to identify gene mutations beyond KRAS that predict response to panitumumab in a randomized phase III monotherapy study of metastatic colorectal cancer. *American Association for Cancer Research Annual Meeting*; Washington, DC, USA; April 17-21, 2010. Abstract 174.

90. Rothemberg M, Clarke MF. Cancer stem cells. In: *Essentials of stem cells biology*. 2nd Edition. Edited by Lanza R. Chapter 53, pp. 469-483. Elsevier, 2009.

91. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; 414: 105-111.

92. Bapat S, Collins A, Dean M et al. Cancer stem cells: similarities and variations in the theme of normal stem cells. In: *Cancer stem cells. Identification and targets*. 2nd Edition. Edited by Bapat S. Chapter 1, pp. 1-26. Wiley, 2009.

93. Maugeri-Saccà M, Vigneri PG, De Maria R. Cancer stem cells and chemosensitivity. *Clin Cancer Res* 2011 May 27. [Epub ahead of print]

94. Hart LS, El-Deiry WS. Invincible, but not invisible: imaging approaches toward in vivo detection of cancer stem cells. *J Clin Oncol* 2008; 26: 2901-2910.

95. Crea F, Duhagon MA, Farrar WL, Danesi R. Pharmacogenomics and cancer stem cells: a changing landscape? *Trends Pharmacol Sci* 2011 Apr 27. [Epub ahead of print]

96. Ricci-Vitiani L, Fabrizi E, Palio E, De Maria R. Colon cancer stem cells. *J Mol Med* 2009; 87: 1097-1104.

97. Zeki SS, Graham TA, Wright NA. Stem cells and their implications for colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2011; 8: 90-100.

98. Ricci-Vitiani L, Lombardi DG, Pilozzi E et al. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; 445: 111-115.

99. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; 445: 106-110.

100. Corbeil D, Roper K, Hellwig A et al. The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J Biol Chem* 2000; 275: 5512-5520.

101. Shmelkov SV, Butler JM, Hooper AT et al. CD133 expression is not restricted to stem cells, and both CD133+ and CD133− metastatic colon cancer cells initiate tumors. *J Clin Invest* 2008; 118: 2111-2120.

102. Kemper K, Sprick MR, de Bree M et al. The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation. *Cancer Res* 2010; 70: 719-729.

103. Dalerba P, Dylla SJ, Park IK et al. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA* 2007; 104: 10158–10163.

104. Huang EH, Hynes MJ, Zhang T et al. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res* 2009; 69: 3382-3389.

105. Horst D, Kriegl L, Engel J et al. CD133 expression is an independent prognostic marker for low survival in colorectal cancer. *Br J Cancer* 2008; 99: 1285-1289.

106. Li CY, Li BX, Liang Y et al. Higher percentage of CD133+ cells is associated with poor prognosis in colon carcinoma patients with stage IIIB. *J Transl Med* 2009; 7: 56-63.

107. Artells R, Moreno I, Díaz T et al. Tumour CD133 mRNA expression and clinical outcome in surgically resected colorectal cancer patients. *Eur J Cancer* 2010; 46: 642-649.

108. Lin EH, Hassan M, Li Y et al. Elevated circulating endothelial progenitor marker CD133 messenger RNA levels predict colon cancer recurrence. *Cancer* 2007; 110: 534-542.

109. Saigusa S, Tanaka K, Toiyama Y et al. Correlation of CD133, OCT4, and SOX2 in rectal cancer and their association with distant recurrence after chemoradiotherapy. *Ann Surg Oncol* 2009; 16: 3488-3498.

110. Lugli A, Iezzi G, Hostettler I et al. Prognostic impact of the expression of putative cancer stem cell markers CD133, CD166, CD44s, EpCAM, and ALDH1 in colorectal cancer. *Br J Cancer* 2010; 103: 382-390.

111. Iinuma H, Watanabe T, Mimori K et al. Clinical significance of circulating tumor cells, including cancer stem-like cells, in peripheral blood for recurrence and prognosis in patients with Dukes' stage B and C colorectal cancer. *J Clin Oncol* 2011; 29: 1547-1555.

112. Faltas B, Zeidan A, Peters K et al. Identifying circulating tumor stem cells that matter: the key to prognostication and therapeutic targeting. *J Clin Oncol* 2011 Jun 20. [Epub ahead of print]

113. Crea F, Fornaro L, Masi G et al. Faithful markers of cancer stem cells: is CD133 sufficient for validation in the clinics? *J Clin Oncol* 2011. [in press]

114. Quina AS, Buschbeck M, Di Croce L. Chromatin structure and epigenetics. *Biochem Pharmacol* 2006; 72: 1563-1569.

115. Narlikar GJ, Fan HY, Kingston RE. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* 2002; 108: 475-487.

116. Mathews LA, Crea F, Farrar WL. Epigenetic gene regulation in stem cells and correlation to cancer. *Differentiation* 2009; 78: 1-17.

117. Nephew K, Balch C, Huang T HM et al. Epigenetics in cancer stem cell development. In: *Cancer stem cells. Identification and targets*. 2nd Edition. Edited by Bapat S. Chapter 1, pp. 1-26. Wiley, 2009.

118. Tomlinson I, Bodmer W. Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. *Nat Med* 1999; 5: 11-12.

119. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006; 7: 21-33.

120. Pietersen AM, van Lohuizen M. Stem cell regulation by polycomb repressors: postponing commitment. *Curr Opin Cell Biol* 2008; 20: 201-207.

121. Rajasekhar VK, Begemann M. Roles of Polycomb group proteins in development and disease: a stem cell perspective. *Stem Cell* 2007; 25: 2498-2510.

122. Cao R, Zhang Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Curr Opin Genet Dev* 2004; 14: 155-164.

123. Negishi M, Saraya A, Miyagi S et al. Bmi1 cooperates with Dnmt1-associated protein 1 in gene silencing. *Biochem Biophys Res Commun* 2007; 353: 992-998.

124. Vire E, Brenner C, Deplus R et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006; 439: 871-874.

125. Cao Q, Yu J, Dhanasekaran SM et al. Repression of E-cadherin by the polycomb group protein EZH2 in cancer. *Oncogene* 2008; 27: 7274-7284.

126. Chase A, Cross NC. Aberrations of EZH2 in cancer. *Clin Cancer Res* 2011; 17: 2613-2618.

127. Schlesinger Y, Straussman R, Keshet I et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for *de novo* methylation in cancer. *Nat Genet* 2007; 39: 232-236.

128. Widschwendter M, Fiegl H, Egle D et al. Epigenetic stem cell signature in cancer. *Nat Genet* 2007; 39: 157-158.

129. Cao P, Deng Z, Wan M et al. MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1alpha/HIF-1beta. *Mol Cancer* 2010; 9: 108.

130. Morin RD, Johnson NA, Severson TM et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet* 2010; 42: 181-185.

131. Sneeringer CJ, Scott MP, Kuntz KW et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Natl Acad Sci U S A* 2010; 107: 20980-20985.

132. Lu C, Han HD, Mangala LS et al. Regulation of tumor angiogenesis by EZH2. *Cancer Cell* 2010; 18: 185-197.

133. Köhne CH, Cunningham D, Di Costanzo F et al. Clinical determinants of survival in patients with 5-fluorouracil-based treatment for metastatic colorectal cancer: results of a multivariate analysis of 3825 patients. *Ann Oncol* 2002; 13: 308-317.

134. Koopman M, Venderbosch S, Nagtegaal ID, et al. A review on the use of molecular markers of cytotoxic therapy for colorectal cancer, what have we learned? *Eur J Cancer* 2009; 45: 1935-1949.

135. Loupakis F, Bocci G, Pasqualetti G et al. Targeting vascular endothelial growth factor pathway in first-line treatment of metastatic colorectal cancer: state-of-the-art and future perspectives in clinical and molecular selection of patients. *Curr Cancer Drug Targets* 2010; 10: 37-45.

136. Dallas NA, Xia L, Fan F, et al. Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. *Cancer Res* 2009; 69: 1951-1957.

137. Klarmann GJ, Hurt EM, Mathews LA, et al. Invasive prostate cancer cells are tumor initiating cells that have a stem cell-like genomic signature. *Clin Exp Metastasis* 2009; 26: 433-446.

138. Todaro M, Francipane MG, Medema JP, Stassi G. Colon cancer stem cells: promise of targeted therapy. *Gastroenterology* 2010; 138: 2151-2162.

139. Tan J, Yang X, Zhuang L et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes Dev* 2007; 21: 1050-1063.

140. Wang CG, Ye YJ, Yuan J et al. EZH2 and STAT6 expression profiles are correlated with colorectal cancer stage and prognosis. *World J Gastroenterol* 2010; 16: 2421-2427.

141. Mimori K, Ogawa K, Okamoto M et al. Clinical significance of enhancer of zeste homolog 2 expression in colorectal cancer cases. *Eur J Surg Oncol* 2005; 31: 376-380.

142. Yoon KA, Gil HJ, Han J et al. Genetic polymorphisms in the polycomb group gene EZH2 and the risk of lung cancer. *J Thorac Oncol* 2010; 5: 10-16.

143. Rhodes DR, Yu J, Shanker K et al. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 2004; 6: 1-6.

144. Bracken AP, Dietrich N, Pasini D et al. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes Dev* 2006; 20: 1123-1136.

145. Eisenhauer EA, Therasse P, Bogaerts J et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; 45: 228-247.

146. Giovannetti E, Mey V, Loni L et al. Cytotoxic activity of gemcitabine and correlation with expression profile of drug-related genes in human lymphoid cells. *Pharmacol Res* 2007; 55: 343-349.

147. Rodriguez S, Gaunt TR, Day IN. Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. *Am J Epidemiol* 2009; 169: 505-514.

148. Graudens E, Boulanger V, Mollard C et al. Deciphering cellular states of innate tumor drug responses. *Genome Biol* 2006; 7: R19.

149. Simon JA, Lange CA. Roles of the EZH2 histone methyltransferase in cancer epigenetics. *Mutat Res* 2008; 647: 21-29.

150. Agherbi H, Gaussmann-Wenger A, Verthuy C et al. Polycomb mediated epigenetic silencing and replication timing at the INK4a/ARF locus during senescence. *PLoS One* 2009; 4: e5622.

151. Tsanou E, Peschos D, Batistatou A et al. The E-cadherin adhesion molecule and colorectal cancer. A global literature approach. *Anticancer Res* 2008; 28: 3815-3826.

152. Natalwala A, Spychal R, Tselepis C. Epithelial-mesenchymal transition mediated tumourigenesis in the gastrointestinal tract. *World J Gastroenterol* 2008; 14: 3792-3797.

153. Kuphal F, Behrens J. E-cadherin modulates Wnt-dependent transcription in colorectal cancer cells but does not alter Wnt-independent gene expression in fibroblasts. *Exp Cell Res* 2006; 312: 457-467.

154. Chikazawa N, Tanaka H, Tasaka T et al. Inhibition of Wnt signaling pathway decreases chemotherapy-resistant side-population colon cancer cells. *Anticancer Res* 2010; 30: 2041-2048.

155. Mitomi H, Fukui N, Tanaka N et al. Aberrant p16((INK4a)) methylation is a frequent event in colorectal cancers: prognostic value and relation to mRNA expression and immunoreactivity. *J Cancer Res Clin Oncol* 2010; 136: 323-331.

156. Crea F, Giovannetti E, Cortesi F et al. Epigenetic mechanisms of irinotecan sensitivity in colorectal cancer cell lines. *Mol Cancer Ther* 2009; 8: 1964-1973.

157. Shen Z, Chen L, Hao F et al. Intron-1 rs3761548 is related to the defective transcription of Foxp3 in psoriasis through abrogating E47/c-Myb binding. *J Cell Mol Med* 2010; 14: 226-241.

158. Zhang W, Winder T, Ning Y et al. A let-7 microRNA-binding site polymorphism in 3'-untranslated region of KRAS gene predicts response in wild-type KRAS patients with metastatic colorectal cancer treated with cetuximab monotherapy. *Ann Oncol* 2011; 22: 104-109.

159. Lipkin SM, Chao EC, Moreno V et al. Genetic variation in 3-hydroxy-3-methylglutaryl CoA reductase modifies the chemopreventive activity of statins for colorectal cancer. *Cancer Prev Res (Phila Pa)* 2010; 3: 597-603.

160. Koong AC, Chauhan V, Romero-Ramirez L. Targeting XBP-1 as a novel anti-cancer strategy. *Cancer Biol Ther* 2006; 5: 756-759.

161. Matsuzaki Y, Fujisawa J, Yoshida M. Identification of transcriptional activation domain of TREB5, a CREB/ATF family protein that binds to HTLV-1 enhancer. *J Biochem* 1995; 117: 303-308.

162. Allagnat F, Christulia F, Ortis F et al. Sustained production of spliced X-box binding protein 1 (XBP1) induces pancreatic beta cell dysfunction and apoptosis. *Diabetologia* 2010; 53: 1120-1130.

163. Protiva P, Hopkins ME, Baggett S et al. Growth inhibition of colon cancer cells by polyisoprenylated benzophenones is associated with induction of the endoplasmic reticulum response. *Int J Cancer* 2008; 123: 687-694.

164. Bracken AP, Kleine-Kohlbrecher D, Dietrich N et al. The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes Dev* 2007; 21: 525-530.

165. Balliet RM, Chen G, Gallagher CJ et al. Characterization of UGTs active against SAHA and association between SAHA glucuronidation activity phenotype with UGT genotype. *Cancer Res* 2009; 69: 2981-2989.

166. Negri FV, Wotherspoon A, Cunningham D et al. Mucinous histology predicts for reduced fluorouracil responsiveness and survival in advanced colorectal cancer. *Ann Oncol* 2005; 16: 1305-1310.

167. Catalano V, Loupakis F, Graziano F et al. Mucinous histology predicts for poor response rate and overall survival of patients with colorectal cancer and treated with first-line oxaliplatin- and/or irinotecan-based chemotherapy. *Br J Cancer* 2009; 100: 881-887.

168. Chang CJ, Yang JY, Xia W et al. EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-β-catenin signaling. *Cancer Cell* 2011; 19: 86-100.